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TITLE: Human CD1d-Restricted Natural Killer T (NKT) Cell Cytotoxicity Against Myeloid Cells

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14. ABSTRACT In MHC class II presentation of peptide antigens, CD4 molecules on T cell surface directly bind MHC class II molecules on antigen presenting cells (APCs) and provide co-stimulation for T cells. However, CD1d-restricted T (NKT) cells recognize lipid and glycolipid antigens presented by CD1d molecules on APCs. It's still unclear whether CD4 molecules are also providing co-stimulatory signal for NKT cell activation by binding MHC class II molecules on APCs. Here we showed that CD4 ligation alone or with CD3 on NKT cell surface induces regulatory signal that may modify primary TCR signaling. The stimulatory or inhibitory nature of this signal depends on the substrate that the ligand is bound. Our data don't support that MHC class II is necessary the ligand of CD4 and it's still unclear whether other unknown CD4 ligands exist on antigen presenting cells (APCs) that constitutively stimulate NKT cells through CD4 molecules. It's been reported that MHC class II and invariant chain (Ii) are physically associated with CD1d, but it's poorly understood whether the association has any functional impact on APCs presenting antigens. Here we observed that expression of MHC class II and Ii on APCs may functionally enhance APCs presenting exogenous glycolipid antigen, GalCer to NKT cells, and this enhancement is due to the increased internalization rate of CD1d molecules from cell surface to intracellular compartments where antigens are facilitated to load into CD1d molecules. On the contrary, MHC class II negative APCs may more stably present endogenous antigens to NKT cells. This may have important implications in vivo because immature dendritic cells (iDCs) are more active than mature dendritic cells (mDCs) in the recycling of many molecules between cell surface and intracellular compartments.					
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Introduction

CD1d-restricted natural killer T cells (NKT cells) are a unique subpopulation of T lymphocytes, that have been shown to be able to promote potent anti-tumor responses in a number of different murine (mouse) cancer models. Little is known about whether they may play a role in preventing or controlling human cancers, and in particular there is little information about whether they can function to kill cancer cells as they arise "in vivo" (in the body). However, it is clear that "in vitro" (in the test tube) NKT cells have the capacity to kill other cells that have a molecule called CD1d on their cell surface. One of the main human cell types that have CD1d on the cell surface are cells of the myeloid lineage. Thus, we believe that NKT cells may have special potential for treating CML, because they may be able to kill these tumor cells, and it may be possible to devise a protocol to specifically stimulate the NKT cells to kill CML tumor cells as they begin to proliferate.

The proposal, "Human CD1d-restricted Natural Killer T (NKT) Cell Cytotoxicity against Myeloid Cells", was designed to first investigate whether human NKT cells can kill myeloid leukemic cells, and whether killing requires the presence of a compound called α -GalCer that specifically stimulates NKT cells. The second goal of this project is to try to gain insight into what factors stimulate NKT cells to kill other cells. Our hypothesis is that NKT cells may require the presence of additional factors than the CD1d molecule to become stimulated to kill other cells, and that one of those factors may be the presence of MHC class II molecules that may interact with CD4 molecules expressed on part of NKT cells.

Body

Aim 1: Evaluate the cytotoxicity of CD1d-restricted NKT cells against myeloid cells:

a. Evaluate cytotoxicity of NKT cell clones against myeloid leukemia cell lines.

CD1d and MHC class II expression on the human myeloid leukemic cell lines THP-1, U937, HL-60 and K562 were analyzed by flow cytometry. The THP-1 cells were found to express low endogenous levels of CD1d positive, and very weak positive staining was observed for U937, whereas the HL-60 and K562 cells appeared totally negative. All four appeared negative for MHC class II (**Figure 1**).

The cytotoxic response of NKT cell clones was evaluated for K562 cells that were transfected with CD1d, compared to the untransfected (CD1d-negative) K562 cell line. No significant cytotoxicity was observed towards the untransfected K562 cells. Although the cytotoxicity on self antigen was very low or negative, presence of α -GalCer significantly increased cytotoxicity for CD1d/K562 cells. However, in comparison with DN NKT cell clones, there was no increased cell killing for CD4+ NKT cells (**Figure 2**). This was also true for other CD4+ and DN clones. On the contrary, CD4 negative clones generally appeared more cytotoxic than CD4 positive, and α -GalCer was required for substantial killing response.

b. Test NKT cell clone cytotoxicity against primary leukemic cells from CML patients.

Not done, because IRB approval was not received from the DOD to collect patient samples.

c. Analyze NKT cells in PBMC samples from CML patients "ex vivo" by flow cytometry.

Not done, because IRB approval was not received from the DOD to collect patient samples.

Aim 2: Evaluate roles of CD4 and MHC class II in NKT cell cytotoxicity

a. Evaluate NKT cell clone cytotoxicity against MHC class II⁺ or - target cell lines.

To establish a system to directly evaluate the role of MHC class II expression in CD1d-dependent cytotoxic responses by NKT cells, we identified a matched pair of cell lines that differ only in MHC class II expression. The 3023 cell line was derived from the human lymphoblastoid cell line 721.174, and expresses MHC class II α but not the β -chain, so there is no functional MHC class II in this cell line. The 2001 cell line was generated from 3023 by transfection of HLA-DR β 7, and thus it expresses cell surface MHC class II heterodimers. CD1d was transfected into these cell lines, and selected to generate the 3023-d and 2001-d cell lines that are both homogeneously positive for CD1d but mis-matched for MHC class II (*refer to **Appendix 4***). Cytokine secretion to the 3023-d and 2001-d cells was analyzed for several NKT cell clones. The cytokine secretion responses appeared correlated to the surface CD1d expression of the cell lines (*refer to **Appendix 4***), and was significantly blocked by anti-CD1d blocking antibody (**Figure 3**).

Next we measured the cytotoxicity of various NKT clones to 3023-d and 2001-d cells. We found that the cytotoxicity on MHC class II⁻ 3023-d was higher than that on class II⁺ 2001-d cells in absence or presence of exogenous antigens (**Figure 4A**). The about 5-fold higher CD1d expression level in 3023-d cells might be responsible for the higher killing in absence of exogenous antigen on 3023-d cells, but it seemed the presence of α GalCer increased the cytotoxicity more on 2001-d cells than that on 3023-d cells regardless of CD4⁺ or DN NKT clones (**Figure 4B**). Consistent with this cytotoxicity result, 3023-d cells stimulated higher autoreactive cytokine secretion by the NKT cell clones in absence of exogenous antigen, but similar or less response to saturated amounts of α GalCer compared with 2001-d cells (**Figure 5**). Result from α GalCer titration experiment further clearly demonstrated the positive effect of MHC class II expression on either CD4⁺ or DN NKT cell cytokine production (*refer to **Appendix 4***). Both the cytotoxicity and the cytokine secretion results suggest that expression of MHC class

II didn't affect APC cells presenting self antigens but significantly increase the presentation of exogenous antigen to NKT cells.

b. Test effect of anti-CD4 and anti-MHC II antibody blocking on cytotoxicity.

To specifically investigate the role of CD4 in the activation of the NKT cell clones, cytotoxicity and cytokine release experiments were performed in the presence of an anti-CD4 antibody (clone RPA-T4) or an isotype-matched negative control. Cytokine production from CD4+ NKT clones induced by either self antigen- or α GalCer-loaded APC cells was significantly blocked by addition of the anti-CD4 antibody (RPA-T4) (**Figure 6A**), and this blockade was seen in both 3023-d and 2001-d cells, or even in a re-direction assay (**Figure 6B**), but not seen when using DN NKT clones, suggesting that it is specifically triggered by anti-CD4 antibody binding to CD4 on CD4+ NKT cells. The blocking effect was more prominent for IFN- γ compared with GM-CSF and IL-4 (**Figure 6C**). The presence of the anti-CD4 antibody (RPA-T4) had no effect on NKT cell killing of either the 3023-d and 2001-d cells (**Figure 6D**). In contrast, cytokine secretion from NKT cells was not affected by presence of anti-MHC class II antibody (clone IVA12) (**Figure 7**). This suggests MHC class II might not be the ligand of CD4.

c. Test effect of ligating CD4 on NKT cell activation by APC-free assays.

We next investigated whether ligation of CD4 molecules on NKT cell surface could deliver a co-stimulatory signal. The presence of anti-CD4 antibody (clone RPA-T4) resulted in a dose-dependent increase in the response of CD4+ NKT cells, but not CD4-negative NKT cells, when the primary TCR signal was induced either by an anti-CD3 antibody or by α GalCer-loaded human CD1d fusion protein (**Figure 8A, B**). Calcium (Ca²⁺) flux results also supported that CD4 ligation with CD3 by cross-linking antibody on NKT cells strengthened or accelerated anti-CD3 antibody-induced Calcium flux (**Figure 9A B, C**). These data suggest that ligation of CD4 molecules on NKT cell surface may induce a signal which can modify TCR signaling. The

observations that cytotoxicity was not affected by the addition of an anti-CD4 mAb, and the effect on cytokine secretion was biased towards inhibition of IFN- γ secretion, suggests that different aspects of NKT cell function are not necessarily bundled together; instead, they are controlled by different intracellular signaling pathways, or have different response hierarchies. CD4-mediated signaling appears more involved in the cytokine secretion response than cytotoxicity, and has a greater effect on IFN- γ secretion than GM-CSF and IL-4.

d. Investigate NKT cell cytotoxicity of fresh myeloid cells; correlate with MHC II expression.

Not done, because IRB approval was not received from the DOD to collect patient samples.

All these results strongly support that ligation of CD4 molecules on NKT cells can introduce regulatory signal to TCR signaling pathway and significantly affect NKT cell cytokine production upon ligation with CD3 molecules or alone. Different aspects of NKT cell function, e.g. various cytokine secretion and cytotoxicity, have different triggering thresholds upon activation. IFN- γ secretion has higher threshold than GM-CSF and IL-4. These data don't support that MHC class II expressed on APC cells is necessary the ligand of CD4 on NKT cells, but it's still unclear whether other unknown ligands expressed by APCs are constitutively stimulating NKT cells through CD4 molecules.

Based on the data we observed that MHC class II expression on APC cells do enhance the activation of NKT cells by exogenous antigen α GalCer while it does not affect the presentation of self antigens. This is only observed by measuring cytokine production but not cytotoxicity. However, this enhancement seems not to be due to the co-stimulation of CD4 ligation with MHC class II, but to the facilitated antigen processing by MHC class II expression. To evaluate the role of MHC class II in CD1d antigen presentation, we designed a series of experiments to analyze the trafficking of CD1d in both MHC class II⁺ (2001-d) and II⁻ (3023-d) APCs, which will be described in detail in **Appendix 4**. (Please refer to **Appendix 4** for the following two parts)

CD1d trafficking affects exogenous lipid antigen presentation.

Invariant chain (Ii) is also involved in MHC class II-associated CD1d antigen presentation.

.Key Research Accomplishments

1. Generation of MHC class II- and class II+ human CD1d transfectants, 3023-d and 2001-d. They are very helpful tools for investigating the role of MHC class II in CD1d antigen presentation;
2. Understanding that CD4 on human NKT cells may constitutively direct regulatory signals (either stimulatory or inhibitory) to primary TCR signaling, by which CD4 molecules may qualitatively rather than quantitatively modify NKT cell function;
3. Understanding that a pool of human CD1d molecules that associate with MHC class II and Ii are facilitated to present exogenous lipid antigen by accelerated CD1d internalization while the MHC class II non-associated CD1d molecules may more steadily present exogenous lipid antigens;
4. This project directly opens up many new and interesting questions, for example, how MHC class II and Ii accelerate CD1d internalization, how CD1d molecules can still traffick to lysosomal compartments even without MHC class II expression in 3023-d cells and what's the effect of CD1d trafficking through different intracellular compartments on presentation of endogenous and exogenous lipid antigens;

Reportable Outcomes

1. A poster for the Annual Meeting of the American Association of Immunologists (AAI) (*Boston MA, May 12-16, 2006*) (see **Appendix 1**).
2. An abstract for Wisconsin Symposium on Human Biology (*Madison WI, May 22-25, 2006*) (see **Appendix 2**).
3. An abstract for University of Wisconsin Immunology Research Symposium (*Madison WI, June 7, 2006*) (see **Appendix 3**).
4. The submitted paper (*Xiuxu Chen, et al, MHC Class II and Invariant Chain Regulate Presentation of Exogenous Antigens by Human CD1d Molecules*) (see **Appendix 4**).

Conclusions

The work that has been done here was funded by Department of Defense (DOD) Fiscal Year 2003 (FY03) Chronic Myelogenous Leukemia Research Program (CMLRP): Exploration–Hypothesis Development Award (EHD). It was proposed to investigate the cytotoxicity of NKT cells either from healthy donors and leukemia patients on both leukemic cell lines and primary leukemic cells, and the role of CD4 in co-stimulating CD1d-restricted NTK cell activation by binding to MHC class II molecules on CD1d+ APCs. We found that CD4 can induce regulatory signals upon ligation, and the stimulatory or inhibitory nature of these signals is dependent on the substrates that provide the ligands. The IFN- γ -biased inhibition and unaffected cytotoxicity by CD4 signal suggests that various aspects of NKT cell function may have different triggering thresholds upon activation. This finding is important because it suggests CD4 may qualitatively regulate rather than just quantitatively amplify the primary TCR signals that we previously had believed. Our data don't support that MHC class II molecules expressed by APCs are necessary the natural ligands for CD4 molecules on NKT cell surface, but it's still unclear whether other unknown ligands still exist and are constitutively stimulating NKT cells through CD4 molecules. Although we couldn't do any experiment of fresh cells from blood samples from healthy donors and leukemic patients as we proposed, it's still very interesting to know from this project that a pool of human CD1d molecules that associate with MHC class II and Ii molecules significantly facilitate rapid presentation of exogenous lipid antigen while the MHC class II and Ii non-associated CD1d molecules are more steady in presenting exogenous lipid antigens. This may have also important implications in lipid antigen presentation by iDCs and mDCs in vivo during tumor vaccine development for leukemia patients. Further investigation of CD1d trafficking may help us understand how CD1d is evolved to survey various intracellular compartments to control microbial infections while avoid autoimmune diseases.

References

(For references, see **Appendix 4**)

Supporting data

Figure legends

Figure 1 Analysis of CD1d and MHC class II surface expression on myeloid leukemic cell lines by flow cytometry. Filled histograms show specific antibody staining, open histograms show negative isotype controls.

Figure 2 Cytotoxicity of CD4+ and DN NKT cells on myeloid cell lines. Specific killing of CD4+ (J24L.10) and DN (J24N.70) NKT clones on myeloid K562 cells was analyzed either with self-antigen (open bars) or exogenous antigen α GalCer (filled bars). APCs were pulsed with 100ng/ml α GalCer for 16 hours, and after washing, cells were labeled by $100\mu\text{Ci}/5 \times 10^5$ cells/ml chromium-51 (51-Cr) for 4 hours before co-incubation with NKT cells. For self-antigen, no α GalCer was pulsed before 51-Cr labeling. 51-Cr release in supernatant in 4-hour co-incubation was measured by PerkinElmer Microbeta Trilux plate reader, and the specific killing was calculated as following:

$$\text{Specific killing (\%)} = 100\% * (CPM_{\text{target}} - CPM_{\text{spontaneous}}) / (CPM_{\text{max}} - CPM_{\text{spontaneous}})$$

Figure 3 CD1d-dependent cytokine production by NKT cell auto-reactivity. 5×10^4 cells/well 2001-d cells or mock transfected 2001 cells (open bars) were co-incubated with the same amount of NKT cells with RPMI 1640 (dotted bars), isotype control antibody IgM (thatched bars) or mouse anti-human CD1d antibody (clone CD1d59), and incubated for 16 hours at 37°C. Supernatant was collected for ELISA to measure the cytokine concentration. Purified GM-CSF cytokine from *Peprtech* was used to determine the GM-CSF concentration in supernatant.

Figure 4 Specific killing of NKT clones on self-antigen or α GalCer-pulsed 3023-d and 2001-d cells. **A)** Specific killing of a NKT clone (J24L.17) on MHC class II- 3023-d and class II+ 2001-d cells was analyzed either with self-antigen (open bars) or exogenous antigen α GalCer (filled bars). Experiments were done similarly as described in Legend *Figure 3* except that the concentration of α GalCer was 1ng/ml. **B)** Specific killing of CD4+ (J24L.17) and DN (J24N.70) clones on 3023-d and 2001-d target cells induced by 1ng/ml exogenous antigen α GalCer. Data were plotted as fold cytotoxicity increased by α GalCer over autoreactivity.

Figure 5 Cytokine production by three NKT clones in response to saturated α GalCer-pulsed APCs. 3023-d (open bars) and 2001-d (filled bars) cells were pulsed with 50ng/ml α GalCer for 42 hours at 37°C, and then 5×10^4 /well cells were co-incubated with the same amount of NKT cells (clone J3N.4) for 16 hours before harvesting supernatant for cytokine quantification by ELISA.

Figure 6 Cytokine production but not cytolysis was blocked by anti-CD4 antibody in presence of α GalCer-loaded APCs. **A)** 5×10^4 /well 5ng/ml α GalCer-loaded 3023-d or 2001-d cells and the same amount of either CD4+ (clone J3N.4) or DN (J24N.70) NKT cells were co-incubated in presence of 10 μ g/ml anti-CD4 antibody (RPA-T4) (filled bars) or mIgG1 isotype control (P3) (open bars) for 16 hours at 37°C. Supernatant was harvested and cytokine secretion was measured by commercial ELISA kit. **B)** Re-direction assay: hCD1d/P815 cells were used as APCs for antigen presentation assay. Logarithmically growing hCD1d/P815 cells pulsed with indicated concentrations of α GalCer were first bound by either anti-CD4 antibody (RPA-T4) (solid lines) or mIgG1 isotype control (P3) via Fc γ receptors for 30 minutes on ice, and after being washed twice, 5×10^4 /well cells were co-incubated with same amount of either CD4+ (clone J24N.22 and clone J3N.4) or DN (clone J24N.70) NKT cells for 16 hours at 37°C. Supernatant was collected for cytokine production by ELISA. **C)** Anti-CD4 antibody showed unequal inhibition on various cytokines. Similar re-direction assay was done by using hCD1d/P815 cells pulsed with 50ng/ml α GalCer. Clone J24N.22 was used for its capability of

producing various cytokines. GM-CSF, IL-4 and IFN- γ in supernatant were analyzed by ELISA.

D) Cytotoxicity of CD4⁺ NKT cells (clone J24L.17) on both 3023-d and 2001-d cells was not affected by presence of anti-CD4 antibody in comparison with control by 51-Cr release assay. Experiment was performed the same as described in the Legend of Figure 2.

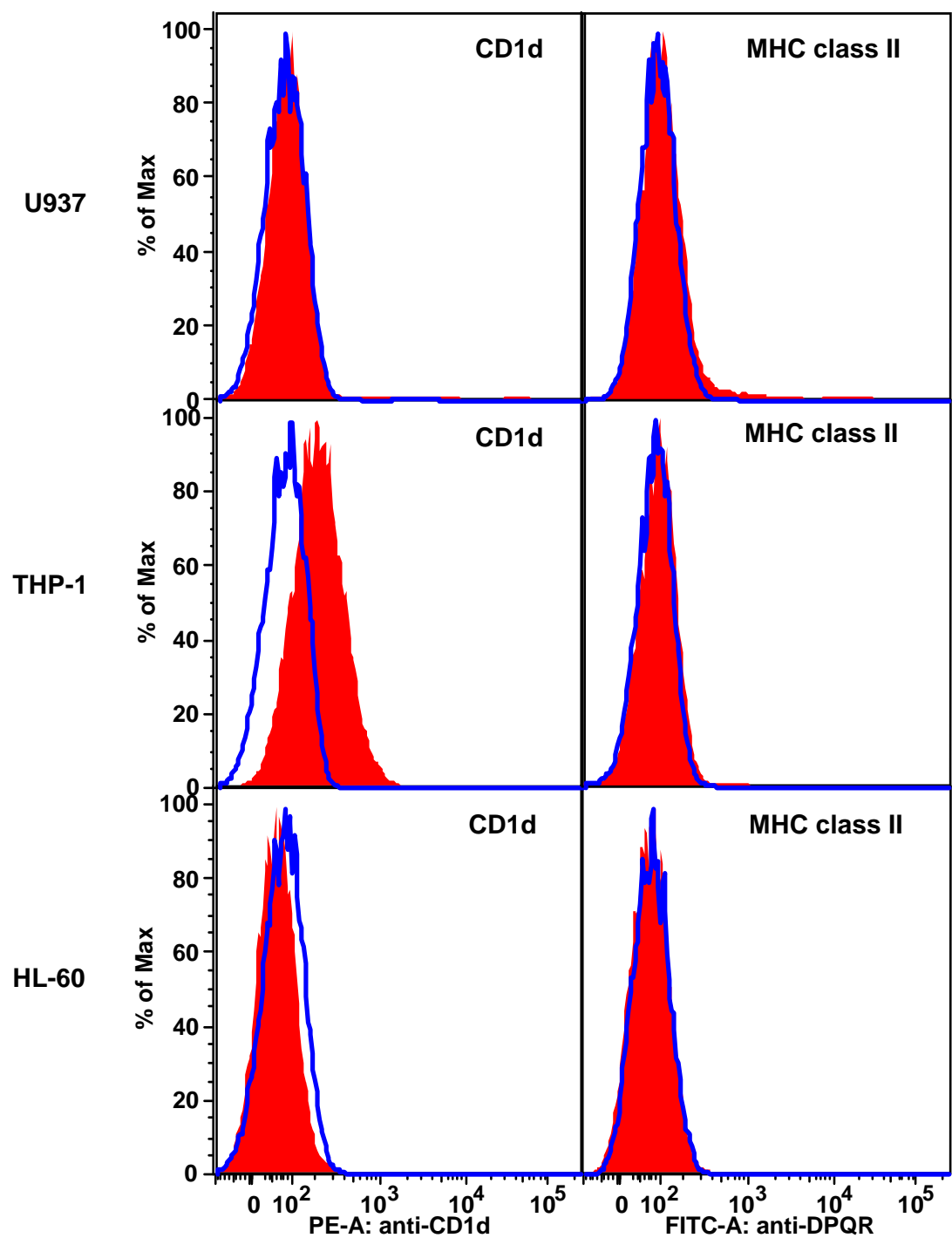
Figure 7 Anti-MHC class II antibody didn't affect cytokine secretion from either CD4⁺ or DN NKT cells in response to MHC class II- or II+ APCs. APCs pulsed by 5ng/ml α GalCer for 16 hours at 37°C was first labeled by anti-MHC class II antibody (clone IVA12) or mIgG1 isotype control (MOPC-21) for 30 minutes on ice, then they were co-incubated with the same amount of CD4⁺ (clone J3N.4) or DN (clone J24N.70) NKT cells (5×10^4 /well) for 16 hours at 37°C. Supernatant was harvested for cytokine production by ELISA.

Figure 8 Enhancement of GM-CSF production by CD4 polarization on CD4⁺ NKT cell clones by plate-bound anti-CD4 antibody. **A)** 100ng/ml anti-CD3 antibody (SPVT-3b) alone, or together with indicated concentrations of mIgG1 (clone MOPC-21) (triangle) or anti-CD4 antibody (clone RPA-T4) (square) were coated on 96-well plates at 4°C overnight. Following coating, plates were blocked with 10mg/ml PBS/BSA for 2 hours at room temperature, and then 5×10^4 /well NKT cells (clone J24N.22) were incubated for 16 hours at 37°C before harvesting supernatant for cytokine quantification by ELISA. **B)** 50 μ l/well 50ng/ml α GalCer with 10 μ g/ml human CD1d-Fc fusion protein were added alone, or together with indicated concentrations of mIgG1 (MOPC-21) (triangle) or anti-CD4 antibody (RPA-T4) (square), and incubated at 37°C overnight.

Figure 9 Effects of CD4 cross-linking on anti-CD3 antibody-induced NKT cell Ca²⁺ flux. 4×10^5 CD4⁺ (clone J24L.17) or DN (clone J3N.1) NKT cells were labeled with 1 μ M Fluo-4 and 2 μ M Fura Red in PBS for 50 minutes at 30°C. After being washed once, cells were further incubated for another 30 minutes at 30°C. **A)** Anti-CD4 antibody (RPA-T4) (red and green curves) or mIgG1 isotype control (MOPC-21) (blue curve) with final concentrations of 1 μ g/ml in 1mg/ml PBS/BSA were added and pre-incubated for 10 minutes at room temperature before addition of

anti-CD3 antibody. Then, cells were acquired at the indicated time points. After 8-10 minutes, 10µg/ml secondary cross-linking antibody (goat anti-mouse IgG as shown by solid arrow) was added to the rest cell suspension and the time course was continued up to 16-20 minutes. **B)** Anti-CD4 antibody (RPA-T4) (red and green curves) or mIgG1 isotype control (MOPC-21) (blue curve) was added together with 0.5µg/ml anti-CD3 antibody (red and blue curves) or vehicle PBS/BSA buffer (green curve), and all the following steps were the same as above. **C)** DN NKT clone (J3N.1) was also analyzed the same way as the CD4+ NKT clone tested above.

Figure 1



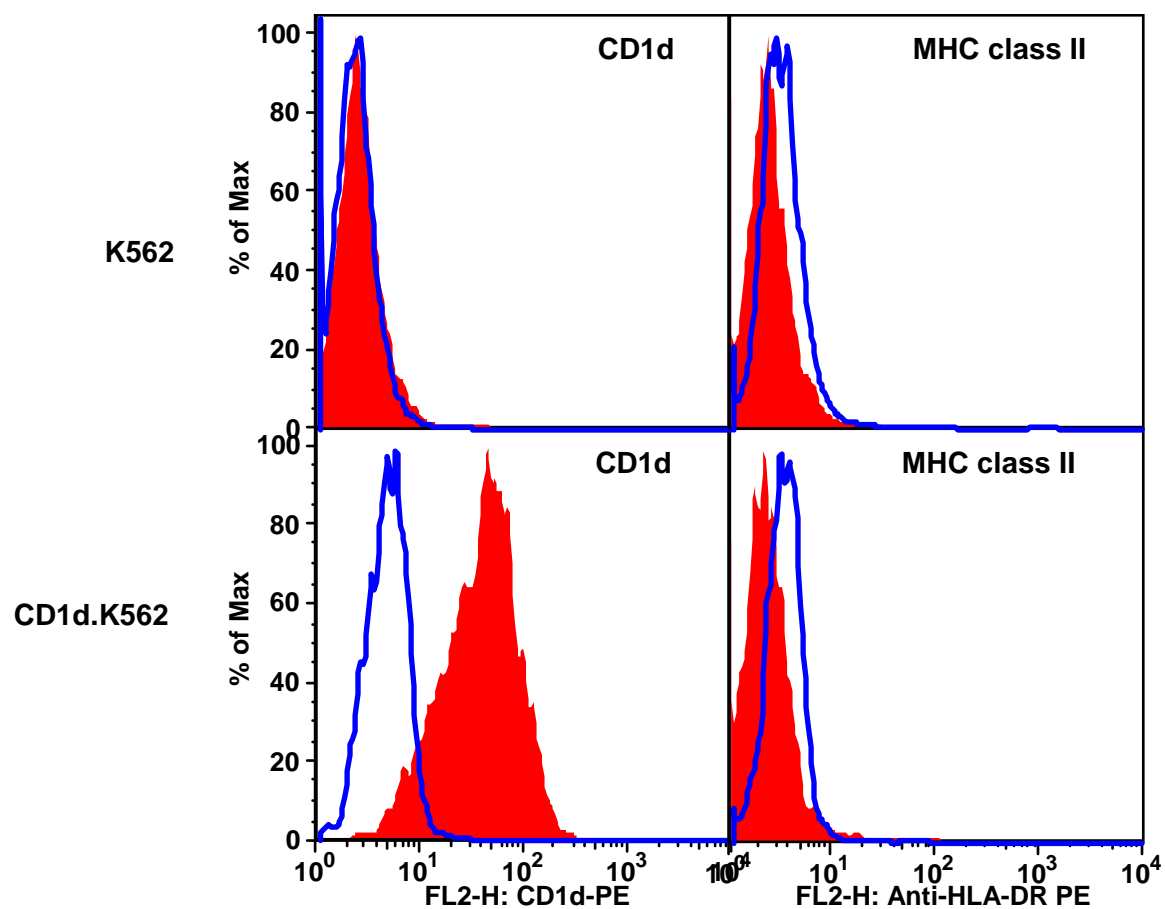


Figure 2

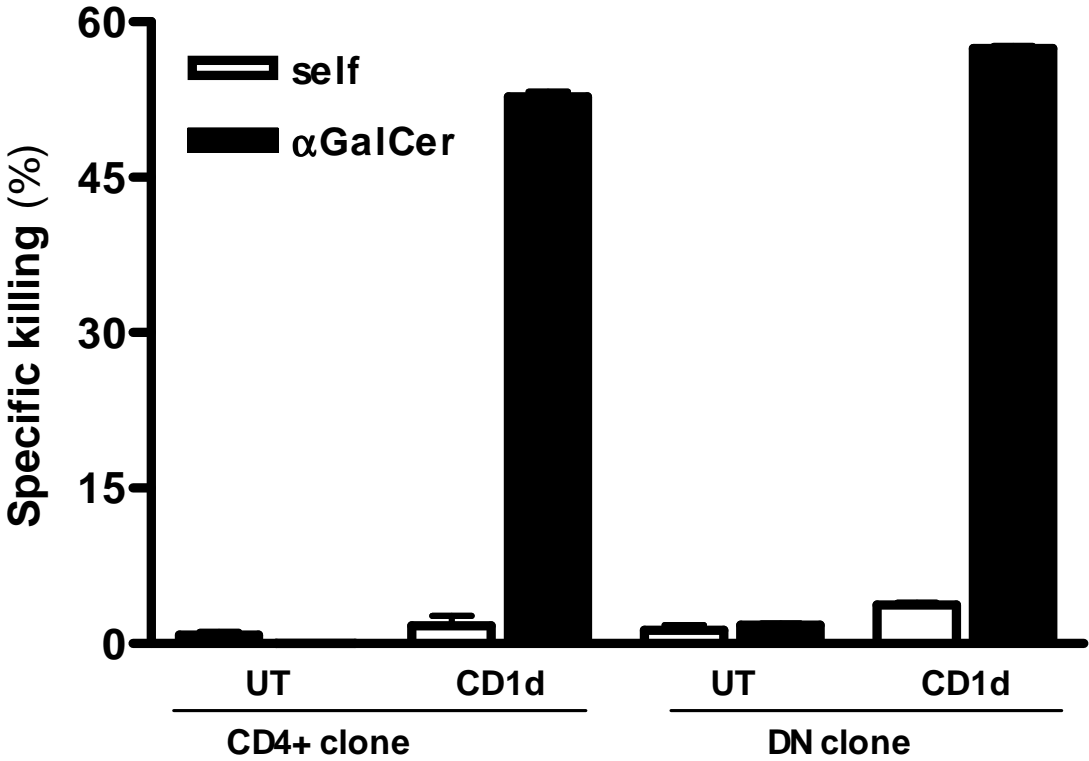


Figure 3

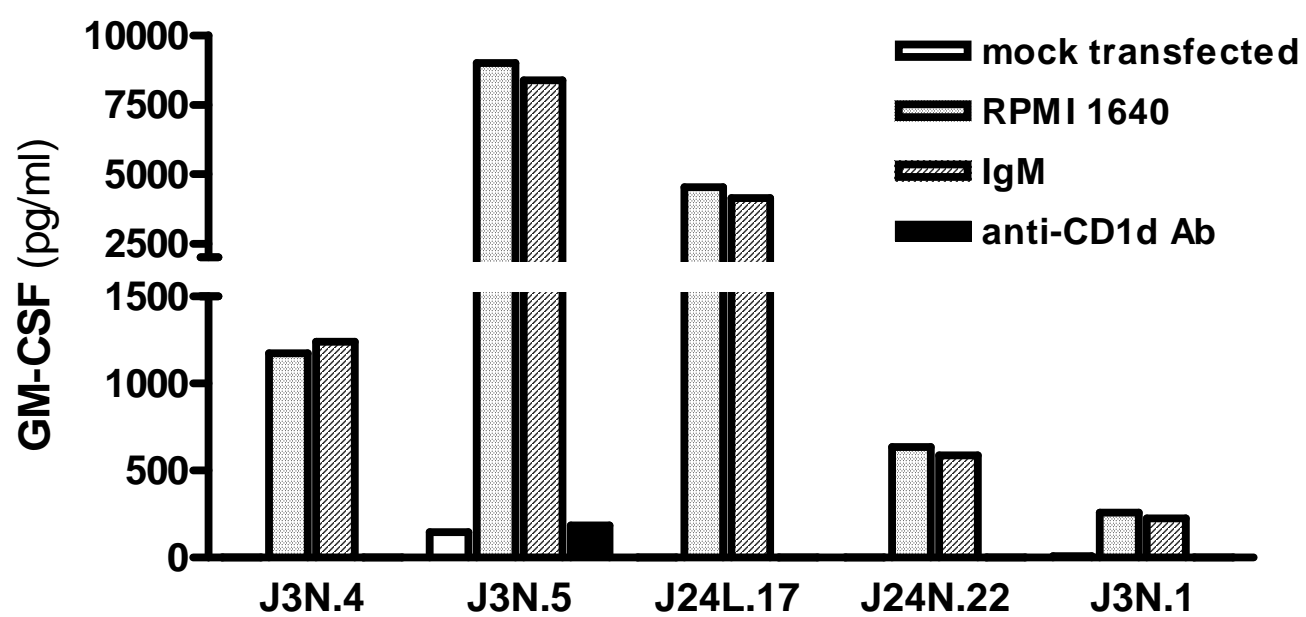


Figure 4

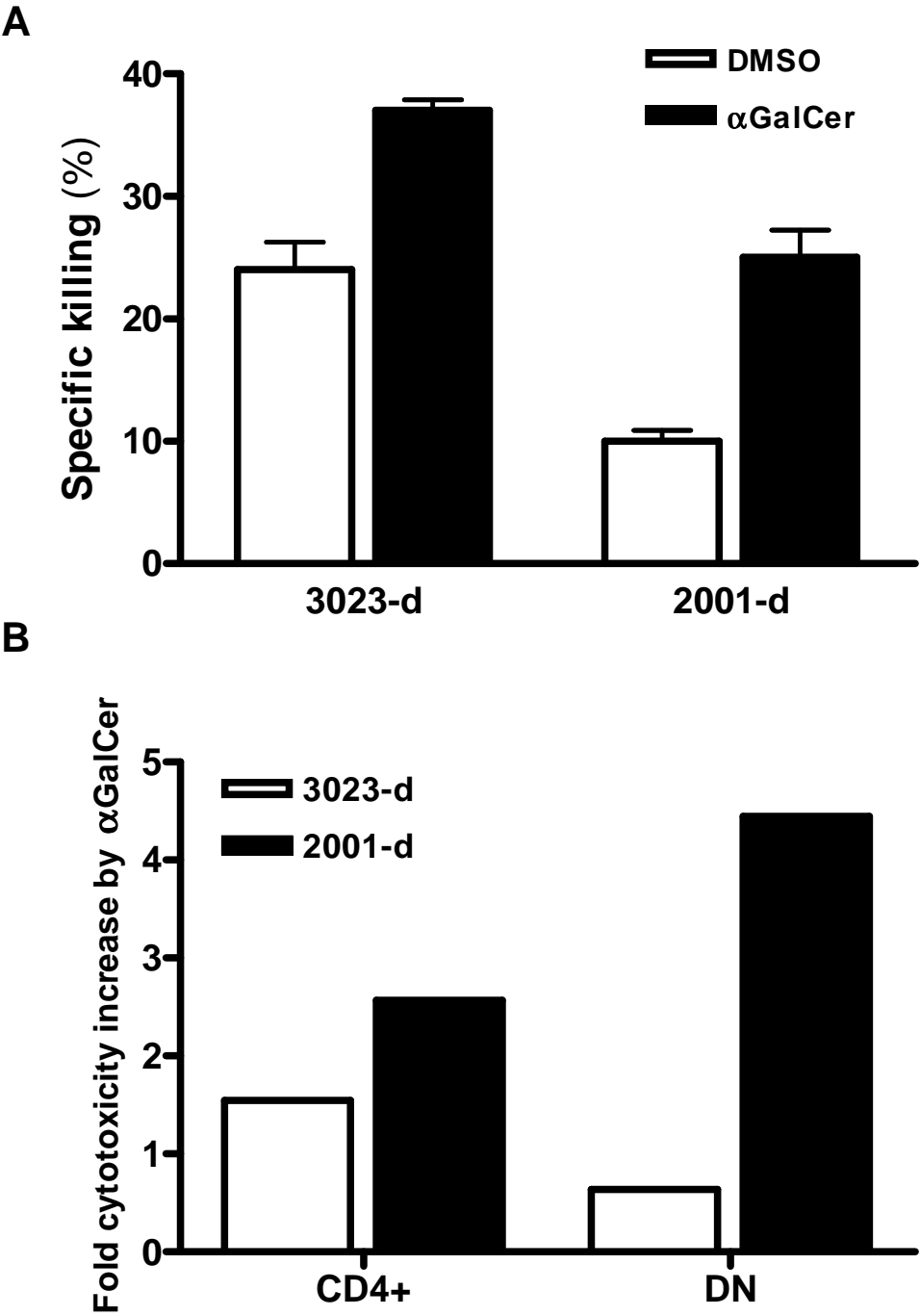


Figure 5

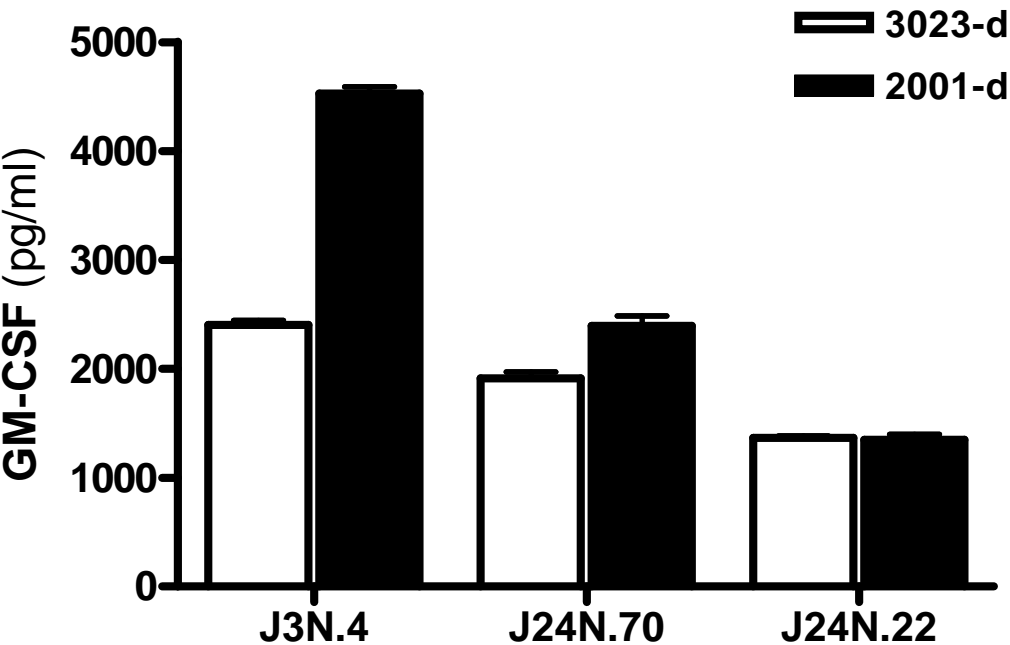


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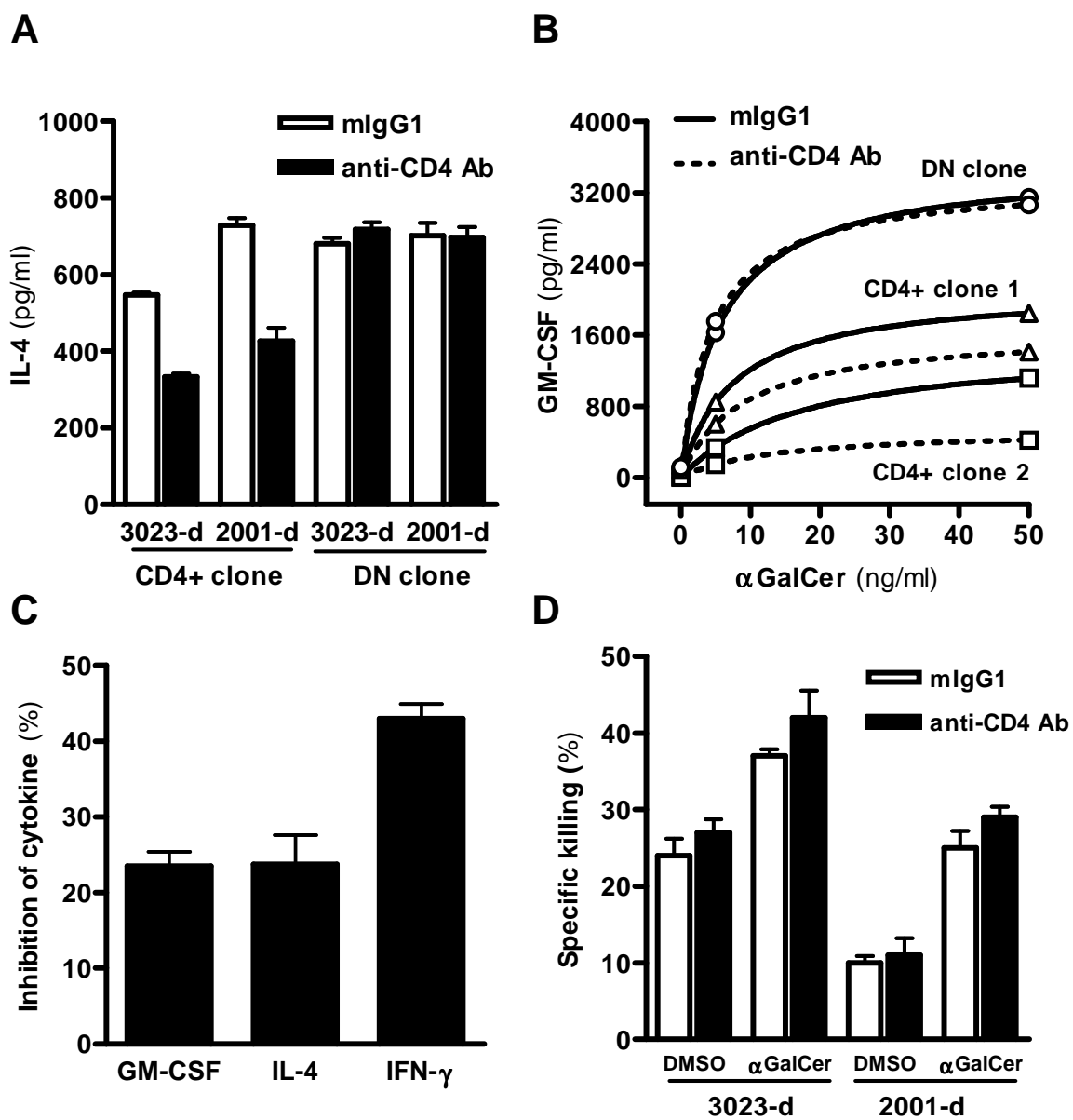


Figure 7

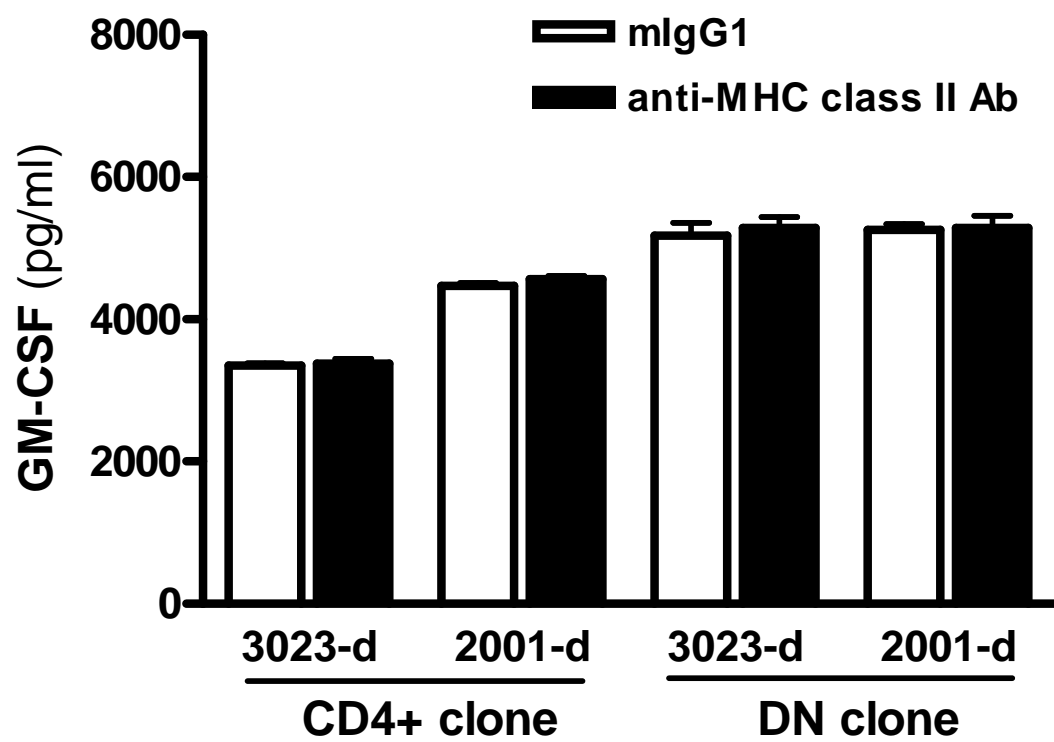


Figure 8

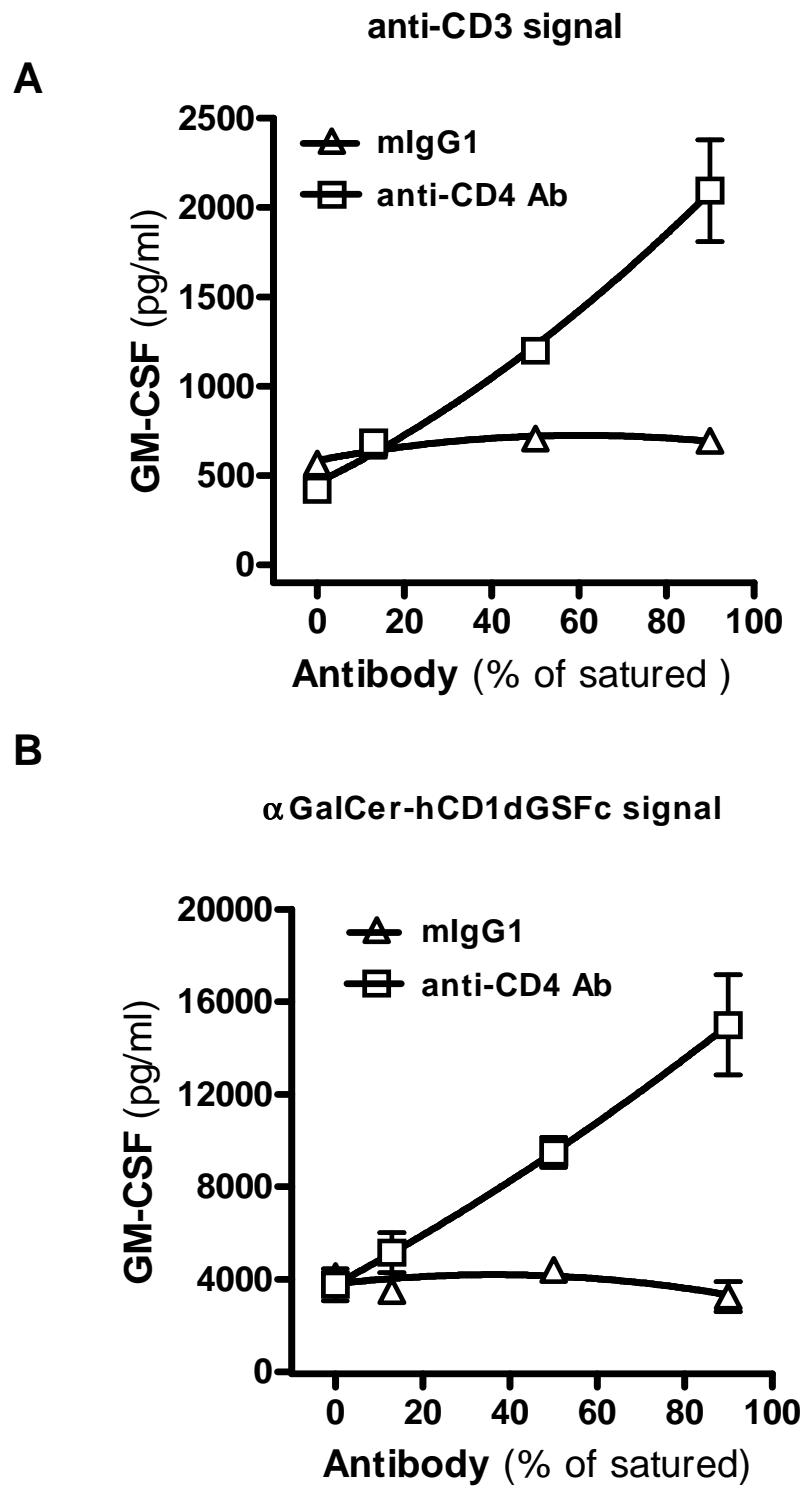
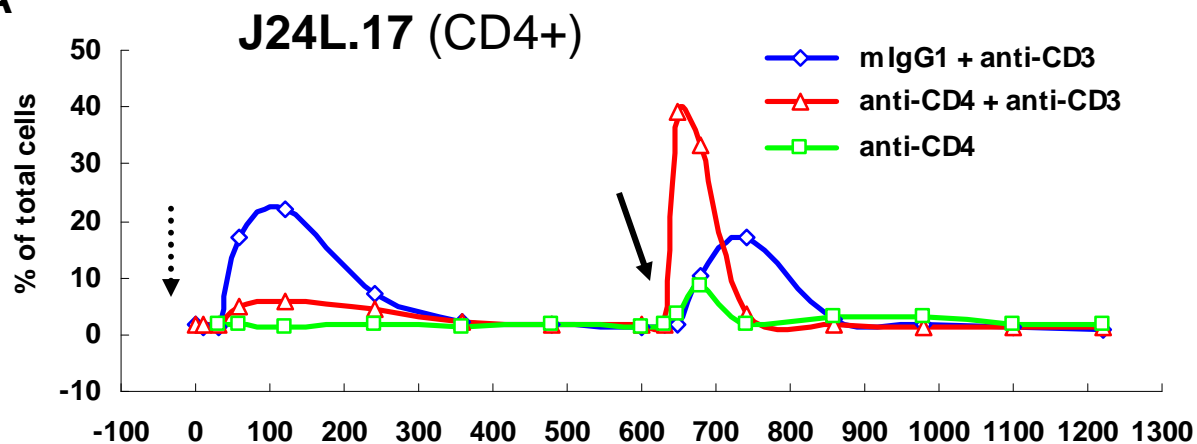
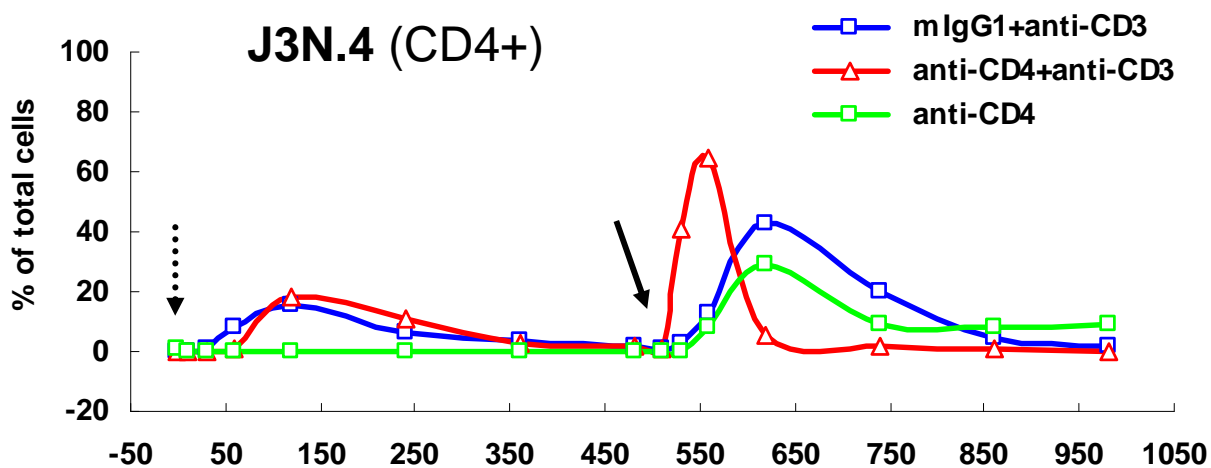


Figure 9

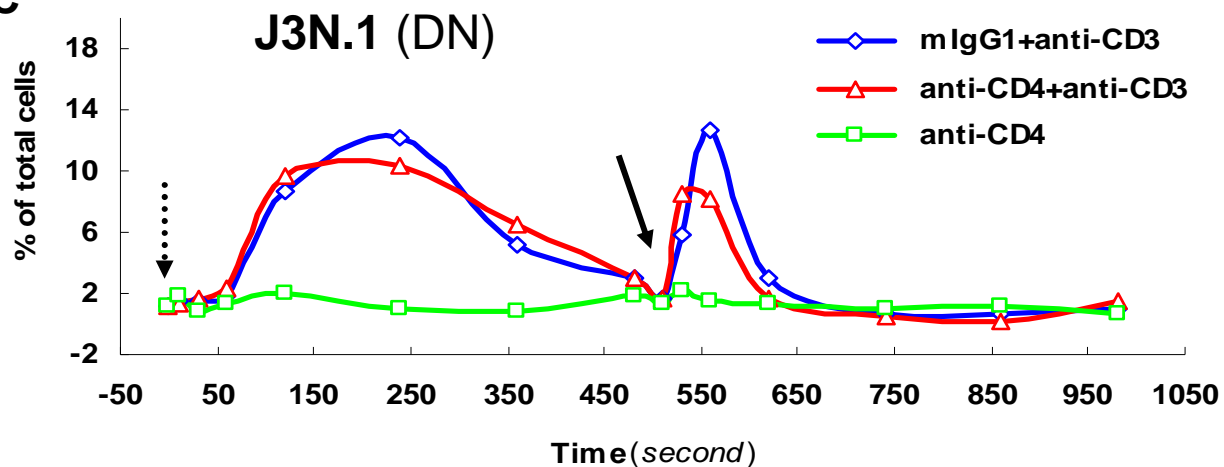
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Appendices

Appendix 1 A poster for the Annual Meeting of the American Association of Immunologists (AAI) (*Boston MA, May 12-16, 2006*).

MHC class II expression functionally enhances CD1d presentation of an exogenous glycolipid by accelerating CD1d internalization

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CD1d molecules present self and foreign lipids to CD1d-restricted NKT cells. Recent data suggest that lipid antigen loading is heavily influenced by the intracellular trafficking of CD1d molecules. Previous studies have shown that a fraction of CD1d molecules co-traffick with MHC class II molecules and the invariant chain from the ER to the plasma membrane. However, it remains unclear whether the presence of MHC class II molecules functionally affects CD1d antigen presentation and NKT cell activation. Here we show that MHC class II expression markedly enhances the kinetics of presentation of the exogenous antigen alpha-galactosylceramide (alpha-GalCer) to human NKT cells, but does not seem to affect their responses to self-antigens. Presentation of a di-galactose ceramide, GalGalCer, that requires lysosomal processing for recognition by NKT cells, appeared similar in MHC class II⁺ and II⁻ cells, suggesting that CD1d molecules could traffic to lysosomes in both cases. Surprisingly, CD1d molecules appeared to be internalized from the cell surface more rapidly in MHC class II⁺ cells, suggesting that the functional difference in antigen presentation may result from faster CD1d recycling. These results suggest that the fraction of CD1d molecules that traffick with MHC class II molecules are important in loading exogenous antigens, and may have implications for exogenous antigen presentation in cells that differ in MHC class II trafficking such as immature and mature dendritic cells.

Supported by DOD Hypothesis Development Award CM030014

Appendix 2 An abstract for Wisconsin Symposium on Human Biology (*Madison WI, May 22-25, 2006*).

MHC class II expression functionally enhances CD1d presentation of an exogenous glycolipid by accelerating CD1d recycling

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NKT cells are a specialized subset of T cells that may play an important role in immune responses to tumors. NKT cells recognize self and foreign lipids presented by CD1d glycoproteins that are expressed on antigen presenting cells. The loading of exogenous lipid antigens into CD1d molecules is thought to occur mainly in late endosomal intracellular compartments, but little is known about the mechanisms that control the intracellular trafficking of human CD1d molecules through the endosomal vesicular system. Previous studies have shown that a fraction of CD1d molecules co-traffick with MHC class II molecules and the invariant chain, but the functional impact of this observation remains unclear. Here we show that MHC class II expression markedly enhances the kinetics of presentation of an exogenous lipid antigen called alpha-galactosylceramide (α -GalCer) to human NKT cells. This effect is due to more rapid CD1d recycling between the cell surface and intracellular compartments in MHC class II⁺ cells than in MHC class II⁻ cells, which appears to allow more rapid antigen loading in MHC class II⁺ cells. In contrast, antigen presentation is more stable in MHC class II⁻ cells than in class II⁺ cells, consistent with the observation that CD1d molecules are not as rapidly internalized in these cells. These results suggest that the fraction of CD1d molecules that traffick with MHC class II molecules are specialized for rapid loading of exogenous antigens, whereas the duration of CD1d-mediated antigen presentation is improved in the absence MHC class II trafficking. These findings have important implications for CD1d-mediated presentation of exogenous antigens in human dendritic cells (DCs), as MHC class II molecules are known to traffick actively in immature dendritic cells and are nearly completely static in mature DCs.

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Appendix 3 An abstract for University of Wisconsin Immunology Research
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MHC class II expression functionally enhances CD1d presentation of an exogenous glycolipid by accelerating CD1d internalization

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NKT cells are a specialized subset of T cells that can potently stimulate tumor rejection. The functional responses of NKT cells are controlled by recognition of lipid antigens presented by CD1d molecules. Recent data suggest that loading of lipids into CD1d molecules mainly occurs in intracellular compartments, and is therefore dependent on CD1d intracellular trafficking. Previous studies have shown that a fraction of CD1d molecules co-traffick with MHC class II molecules and the invariant chain from the ER to the plasma membrane, but the functional significance of this association has remained unclear. Using MHC class II⁺ and -negative cells transfected with CD1d, we show that MHC class II co-trafficking markedly influences CD1d-mediated presentation of the synthetic lipid antigen α -galactosylceramide (α -GalCer). Knockdown of the invariant chain using siRNA resulted in a reduction of CD1d surface expression in MHC II⁺ APCs, but not in MHC II⁻ cells, demonstrating that CD1d is associated with the invariant chain in the presence of MHC II molecules. CD1d molecules were internalized from the cell surface more rapidly in MHC II⁺ APCs than in MHC II⁻ cells, and MHC II⁺ cells were more efficiently able to present α -GalCer to human NKT cells. Hence, rapid CD1d recycling from the cell surface due to association with MHC II and the invariant chain appears to promote efficient loading of exogenous lipids. In contrast, CD1d cell surface expression was more stable in MHC class II⁻ cells, and presentation of α -GalCer was prolonged. These results suggest that the fraction of CD1d molecules that traffick with MHC class II and invariant chain molecules are specialized for rapid loading of exogenous lipids, whereas the duration of antigen presentation is improved in the absence MHC class II trafficking. These findings have important implications for CD1d-mediated presentation of exogenous antigens in human dendritic cells (DCs), as MHC class II molecules are known to traffick intracellularly with the invariant chain in immature dendritic cells, and are nearly completely static at the cell surface in mature DCs.

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Appendix 4 The submitted paper (*Xiuxu Chen, et al, MHC Class II and Invariant Chain Regulate Presentation of Exogenous Antigens by Human CD1d Molecules*).

(next page)

Classification: Biological Sciences, Immunology

**MHC Class II and Invariant Chain Regulate Presentation of Exogenous Antigens by
Human CD1d Molecules**

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Abstract

CD1d-mediated presentation of the synthetic lipid α -GalCer potently stimulates NKT cells, resulting in profound immunological activation. The factors that influence uptake and presentation of lipid antigens by human CD1d molecules remain poorly understood. Previous studies have demonstrated that a fraction of CD1d molecules associate with MHC class II molecules and the invariant chain (Ii) during intracellular trafficking, but the functional impact of this association is not known. Here we show that CD1d-mediated presentation of α -GalCer is markedly more efficient in the presence of MHC class II and Ii, whereas the autoreactive responses of human NKT cells to cellular antigens are not increased. A glycolipid that requires lysosomal cleavage for recognition by NKT cells was presented by both MHC class II⁺ and –negative antigen presenting cells (APCs), indicating that CD1d molecules could access lysosomal antigens in both cases. However, CD1d molecules were internalized from the cell surface more rapidly in MHC class II⁺ APCs, and knock-down of the Ii chain by siRNA resulted in slower CD1d internalization in these cells. Thus, association of CD1d with MHC class II and Ii complexes permits rapid entry into the endosomal system. These results suggest that the fraction of human CD1d molecules that traffic with MHC class II and Ii is specialized for efficient uptake of extracellular antigens, while the remaining CD1d molecules preferentially load intracellular antigens.

Introduction

The CD1 family of antigen presenting molecules binds lipids, glycolipids, and lipopeptides, and presents these at the cell surface for recognition by T cells (1). There are five CD1 isoforms, named CD1a-e (2). All five are present in humans, whereas mice and rats have apparently undergone an ancient genetic deletion event that resulted in the loss of all but the CD1d isoform (3). CD1d molecules are required for the selection and function of an evolutionarily conserved subset of T cells, called Natural Killer T (NKT) cells (4). NKT cells are regulatory T cells that can potentially influence immune responses when activated by recognition of specific lipids presented by CD1d (5). Hence, NKT cells and the lipids they recognize have attracted significant interest as potential targets of therapeutic approaches. Lipids derived from both extra- and intracellular sources are known to serve as antigens for NKT cells (6), but the cellular processes that affect lipid binding and presentation by CD1d remain poorly understood.

CD1d molecules contain a deep hydrophobic binding cleft that sequesters lipid alkyl chains, leaving the polar head group more exposed at the surface of the molecule (7, 8). Under physiological conditions, insertion of lipids into the CD1d binding site probably occurs mainly in intracellular compartments, where it is facilitated by specific lipid transfer proteins such as the microsomal transfer protein (MTP) in the endoplasmic reticulum (ER), and the saposins in the lysosome (9-11). As CD1d molecules can accommodate lipids with a variety of different head groups and alkyl chain compositions, but lipid transfer proteins have specificity for particular types of lipids, interaction with these proteins probably substantially influences what lipids are bound by CD1d molecules. Additionally, in some cases, T cell recognition cannot occur unless glycolipid antigens are processed by lysosomal glycosidases to remove interfering glycans (12).

Thus, CD1d antigen presentation is critically affected by trafficking to intracellular sites that contain the accessory proteins required for lipid loading and processing.

CD1d molecules are synthesized in the ER and then follow the secretory pathway through the Golgi to the cell surface (13). Trafficking of CD1d molecules through the endosomal vesicular system is accomplished by association with adaptor proteins that mediate re-internalization from the cell surface and direct localization to endosomal compartments. Four adaptor protein complexes (AP-1,-2,-3,-4) have been identified that bind to tyrosine or di-leucine amino acid motifs in the cytoplasmic tails of transmembrane proteins (14). The cytoplasmic tail sequences of human and murine CD1d molecules both contain tyrosine-based motifs that mediate binding to the AP-2 complex, but the murine CD1d sequence also binds to the AP-3 complex, whereas the human CD1d sequence does not (15-17). The AP-2 complex mediates internalization from the cell surface into early endosomes, and the AP-3 complex directs trafficking from early endosomes to lysosomes (14). Thus, the difference between human and murine CD1d in AP-3 binding may be related to the observation that murine CD1d molecules show steady-state intracellular localization almost exclusively in lysosomal compartments, while human CD1d is distributed in a variety of endosomal vesicles with only a fraction localizing to lysosomes (18).

The intracellular trafficking of CD1d molecules is also affected by association with MHC class II and invariant chain (Ii) molecules (19, 20). MHC class II molecules are heterodimers of an α and β subunit, and associate in the ER with Ii in a nonameric complex consisting of three $\alpha\beta$ dimers and three Ii molecules (21). Association of MHC class II with Ii directs its trafficking to lysosomes, where Ii is cleaved and the MHC class II molecules are subsequently transported to the cell surface (21). A fraction of human CD1d molecules have been shown to associate with

MHC class II and Ii, whereas murine CD1d molecules were found to directly bind Ii (19, 20). Both murine and human CD1d appeared to complex with MHC class II/Ii in the ER and co-traffic to other sites including the cell surface and lysosomes (19, 20).

The functional impact of CD1d association with MHC class II and Ii remains unclear. Expression of Ii was sufficient to reconstitute lysosomal delivery of human and murine CD1d cytoplasmic tail deletion mutants (19, 20). Presentation of lipids that stimulate NKT cell autoreactive responses by murine CD1d appeared to be enhanced by Ii (20), but the Ii chain was not sufficient to compensate for deficiencies in auto-antigen presentation of murine CD1d tail deletion mutants in vivo (22), suggesting that intrinsic cytoplasmic motifs are also critical. As human CD1d does not bind the lysosomal targeting adaptor protein AP-3, association with MHC II and Ii may have a distinct influence on its antigen presenting function. Here we investigate the effects of MHC class II and Ii expression on human NKT cell responses to auto-antigens and α -galactosylceramide (α -GalCer), a potent extracellular antigen (23).

Results

To investigate the effect of MHC class II expression on CD1d-mediated antigen presentation, we generated transfected antigen presenting cells (APCs) that expressed CD1d in the presence of Ii and MHC class II α and β chains, or the α chain alone. The human B-lymphoblastoid cell line 721.174 is a deletion mutant that lacks functional MHC class II α and β chains, but expresses Ii (24). This cell line was transfected with DNA encoding HLA-DR α to generate the 3023 cell line, and 3023 was further transfected with HLA-DR β 7 to generate the 2001 line (25). The resulting cell lines express equivalent amounts of intracellular Ii, and 2001 expresses cell surface HLA-DR, but 3023 does not (see Figure 1A). We stably transfected the

3023 and 2001 cell lines with cDNA encoding human CD1d, generating cell lines called 3023-d and 2001-d. Both are positive for CD1d, but the 3023-d line expresses approximately 5-fold more cell surface CD1d than 2001-d (Figure 1A). This difference appeared to be due to transcription of the CD1d transgene, rather than the difference in HLA-DR expression, as the 3023-d cell line also expresses 5-fold more CD1d mRNA (Figure 1B).

Human CD1d-restricted NKT cell clones were tested for autoreactive responses to cellular lipids presented by CD1d on the 3023-d and 2001-d lines. There was no detectable cytokine production by the NKT cell clones in response to the untransfected 3023 and 2001 cell lines (data not shown), whereas the 2001-d and 3023-d cell lines elicited clearly detectable responses, and the amount of cytokine secretion appeared directly proportional to the level of CD1d cell surface expression on the APC (Figure 2A). These results suggested the NKT cell autoreactive responses were not enhanced by the HLA-DR expression of the 2001-d cell line. In contrast, the 2001-d line was more efficient than the 3023-d line in presenting the exogenous lipid α -GalCer to NKT cell clones (Figure 2B and C). The concentrations of lipid antigen required to stimulate α -GalCer dependent NKT cell responses were approximately 10-fold lower for the 2001-d line than the 3023-d line (Figure 2B), and 2001-d cells were also able to take up and present α -GalCer faster than 3023-d cells (Figure 2C). Notably, the 2001-d cells showed enhanced presentation of α -GalCer to both CD4⁺ and CD4⁻ NKT cell clones (Figure 2B and C). These data suggested the effect was not due to co-stimulation of the NKT clones by HLA-DR, since the enhanced responses were specific to exogenously added antigen and did not require CD4 expression by the NKT cells.

An alternative explanation is that the presence of HLA-DR in the 2001-d line facilitates transport of CD1d molecules to lysosomes, where loading of exogenous lipids is more efficient.

We used confocal microscopy to investigate whether there were detectable differences in lysosomal localization of CD1d in the 2001-d and 3023-d lines. The permeabilized cells were stained for CD1d (red), and LAMP-1 (green) as a marker of lysosomes (Figure 3A). In both cell lines, a fraction of the CD1d immunofluorescence overlapped with the LAMP-1 staining, and there did not appear to be clear-cut differences in the amount of co-localization (Figure 3A). We next investigated the ability of the two cell lines to present the Gal(α 1-2)GalCer glycolipid (GalGalCer), which requires cleavage of the terminal galactose by a lysosomal endoglycosidase before it can be recognized by NKT cells (12). Analysis of NKT cell responses to plate-bound recombinant CD1d molecules loaded in vitro with GalGalCer confirmed that this antigen was not recognized, whereas α -GalCer (the processed form) was able to stimulate NKT cell responses in this system (Figure 3B). Both the 2001-d and the 3023-d cell lines presented GalGalCer to NKT cells, and the antigen-dependent responses were completely blocked by chloroquine, which neutralizes lysosomal acidification (Figure 3C). Chloroquine treatment did not significantly reduce the autoreactive responses of the NKT clones to either 2001-d or 3023-d (Figure 3D), demonstrating that it did not inhibit CD1d-mediated presentation of auto-antigens. These results suggested that the inefficient presentation of exogenous lipid by 3023-d was not due to a defect in CD1d access to lysosomes.

We next investigated whether there was a difference in the rate of CD1d internalization from the cell surface in the 2001-d cells compared to the 3023-d line. Cell surface proteins were biotinylated using a cleavable cross-linking reagent, then the cells were incubated at 37° for varying times to allow CD1d internalization, and the remaining surface biotin was cleaved. The cells were then lysed, and the amount of the biotinylated CD1d (representing the molecules that were protected from biotin cleavage by internalization) was determined by a specific capture ELISA. A higher fraction of the total CD1d consistently appeared biotinylated in the 2001-d

than in the 3023-d cells (Figure 4A), suggesting that the rate of CD1d internalization was faster in these cells. To confirm this, we evaluated cell surface CD1d internalization by flow cytometry. The 2001-d and 3023-d cells were labeled with an unconjugated anti-CD1d mAb, then washed and incubated for varying times as described above to allow internalization, then stained with a fluorescent second-step antibody to detect the remaining labeled CD1d molecules. Consistent with the results from the previous experiment, the cell surface CD1d signal disappeared more rapidly from the 2001-d than the 3023-d cells (Figure 4B), suggesting faster internalization.

In contrast, CD1d appeared to emerge from the endosomal system at the same rate in the 3023-d and 2001-d cells (Figure 4C). The two cell lines were treated with cycloheximide to prevent new protein synthesis, and the CD1d at the cell surface was blocked with unconjugated CD1d42.1 mAb. The cells were then incubated at 37°, and aliquots were removed at varying times and stained with fluorescently labeled CD1d42.1 mAb to detect unblocked CD1d molecules that had newly emerged from the endosomal system. As the rate of exit from the endosomal system appeared similar in the two cell lines, but internalization appeared faster in the HLA-DR⁺ 2001-d cells, these results suggested that association with MHC class II molecules specifically facilitates CD1d entry into the endosomal system from the cell surface.

As association with Ii is responsible for efficient MHC class II trafficking to the endosomal system, we investigated the effect of Ii expression on CD1d. Transient siRNA transfection was used to knock-down protein expression of Ii in the 3023-d and 2001-d lines. From 48-96 hours after transfection the total amount of Ii protein in the siRNA treated 3023-d and 2001-d cells showed about a 50% reduction compared to the amount detectable in cells that were transfected with negative control siRNA, or in untransfected cells (data not shown). The siRNA transfected and control cells were tested for cell surface and total (cell surface +

intracellular) CD1d. The level of cell surface CD1d expression in the 2001-d cells that were transfected with Ii siRNA was diminished by a mean of 31% ($\pm 5.9\%$ standard deviation, $n = 4$ experiments), compared to untreated 2001-d cells (Figure 5A). However, there was no significant reduction in total CD1d expression in the Ii siRNA treated 2001-d cells (Figure 5A). Knock-down of Ii chain expression in the 3023-d cells did not result in reproducibly diminished CD1d cell surface staining (mean reduction = $5\% \pm 7.4\%$, $n = 4$), compared to the control 3023-d cells (Figure 5A). Cells that were transfected with negative control siRNA appeared similar to untreated cells, demonstrating that the effect on the 2001-d cells was specific to the Ii siRNA (Figure 5A). These results suggest that a significant fraction of the CD1d molecules in the 2001-d cells depends on access to Ii for cell surface expression.

We next investigated whether the rate of internalization of the remaining cell surface CD1d molecules was altered in the Ii knocked-down cells. The stability of the cell surface CD1d appeared identical for 3023-d cells that were treated with Ii siRNA, compared to cells that were transfected with the negative control siRNA (Figure 5B). Similar to our previous analyses of untreated 2001-d and 3023-d cells, the 2001-d cells that were treated with the negative control siRNA showed more rapid loss of CD1d cell surface signal than the 3023-d cells (Figure 5B). However, 2001-d cells that were treated with the Ii siRNA showed enhanced stability of CD1d cell surface staining (Figure 5B), suggesting a slower rate of internalization of the remaining cell surface CD1d molecules in these cells. The rate of MHC class I internalization appeared similar for both the 3023-d and 2001-d cell lines, regardless of whether they were transfected with Ii or negative control siRNA (Figure 5C). These results show that in the presence of HLA-DR, the Ii chain specifically influences the kinetics of CD1d cell surface expression, suggesting that association with HLA-DR/Ii complexes promotes rapid CD1d internalization into the endosomal system.

Discussion:

We show here for the first time that HLA-DR and Ii selectively enhance the presentation of extracellular lipids by human CD1d. This effect did not appear to be due to co-stimulation of NKT cells mediated by MHC class II molecules on the APCs, as NKT cell responses to auto-antigens did not appear enhanced, and similar results were obtained from both CD4⁺ and CD4⁻ NKT cell clones. Using biochemical techniques, Kang et al. have previously established that a fraction of human CD1d molecules physically associate with MHC class II and Ii complexes (19). Thus, the enhanced exogenous lipid presentation that we observe seems most likely to be due to direct interaction of MHC class II and Ii complexes with CD1d molecules.

There are several mechanisms by which CD1d interaction with MHC class II and Ii could affect antigen presentation. One possibility is that CD1d binding to MHC II/Ii complexes results in a route of intracellular trafficking that favors loading of exogenous antigens. Our data indicate that association with MHC class II molecules is not required for CD1d to gain access to lysosomal antigens, since both MHC class II⁺ and -negative APCs were able to present GalGalCer to NKT cell clones. Nevertheless, co-trafficking could allow associated CD1d molecules to enter lysosomes by a route that bypasses cell surface expression and early endosomal compartments, as MHC class II and Ii complexes can traffic directly into the endosomal system from the Golgi (21). Such a route might favor loading of exogenous antigens because there would be less opportunity for prior loading of lipids from other compartments. However, our data do not clearly support this trafficking route. We found that knock-down of Ii chain expression resulted in diminished cell surface expression of CD1d in the MHC class II $\alpha\beta^+$ 2001-d cell line, but not in the MHC class II β^- 3023-d line, suggesting that CD1d is complexed with both MHC class II and Ii at the cell surface. As the Ii chain is cleaved by proteases after

entry into the lysosome, it should not affect trafficking after lysosomal delivery, and thus should only affect CD1d cell surface expression if this occurs prior to entry into the lysosome.

Moreover, as Ii chain knock down also resulted in slower CD1d internalization from the cell surface in the 2001-d cells, our data suggest that MHC class II and Ii associated CD1d molecules follow a route of trafficking involving cell surface expression first, then Ii-dependent re-internalization. Therefore, an explanation that is more consistent with our data is that CD1d association with MHC class II and Ii chain complexes leads to more efficient uptake of extracellular antigens by more rapidly moving nascent CD1d molecules into the endosomal system where antigen loading is facilitated by accessory proteins.

Another possibility that is consistent with our data is that association with MHC class II and the Ii chain protects the CD1d molecule from stably binding intracellular lipids, which enables these CD1d molecules to better load extracellular antigens. The Ii knock-down results suggest that binding to MHC class II and Ii complexes rescues a pool of CD1d molecules that otherwise are not able to traffic to the cell surface, perhaps because they did not fold stably or did not associate with an ER lipid. In the absence of MHC class II heterodimers (i.e. in the 3023-d cell line) Ii expression levels did not substantially affect CD1d cell surface expression, which is probably due to the requirement for MHC class II $\alpha\beta$ binding to permit efficient Ii trafficking out of the ER (21). Finally, it is also possible that association with MHC class II molecules recruits CD1d molecules into lipid raft micro-domains that are enriched for co-stimulatory molecules, and that this makes the MHC class II associated CD1d molecules more potently stimulatory to NKT cells.

The observation that NKT cell autoreactive responses do not appear increased in MHC class II⁺ APCs, raises the question of whether loading of intracellular and extracellular antigens occurs separately. Studies of auto-antigen presentation by murine CD1d have shown that

disruption of CD1d lysosomal trafficking or lipid transfer proteins, diminishes NKT cell responses to both exogenous antigens and auto-antigens (10, 26). These findings are consistent with the observation that a self glycolipid called iGb3, that is formed in the lysosome, is critical for the autoreactive responses of murine NKT cells (27). In contrast, previous studies have shown that preventing lysosomal trafficking or antigen loading of human CD1d molecules does not diminish the autoreactive responses of human NKT cells (11, 28). Our data is consistent with this, as chloroquine treatment abrogated presentation of GalGalCer, but did not diminish the autoreactive responses of our NKT cell clones. Thus, intracellular antigens presented by human CD1d may not be loaded in lysosomes.

As the pool of CD1d molecules that associates with MHC class II molecules traffics rapidly and appears specialized for loading exogenous antigens, intracellular antigens may be preferentially loaded by free CD1d molecules that traffic more slowly. This possibility has important implications for CD1d-mediated antigen presentation by dendritic cells (DCs). In immature DCs, MHC class II molecules are prominently localized to intracellular vesicles, whereas upon DC maturation they traffic to the cell surface and remain stably expressed there (29). Hence, uptake of extracellular antigens by CD1d molecules may be more efficient in immature DCs because MHC class II molecules are actively being internalized into lysosomes, whereas upon DC maturation the ability of CD1d to present exogenous antigens may decline with the stabilization of MHC class II at the cell surface, and presentation of intracellular antigens may be favored.

Materials and Methods

Cell lines. The 3023 and 2001 cell lines were a kind gift from Dr. Robert DeMars (25). These cell lines were transfected with cDNA encoding human CD1d in the pCI-puro vector (30), by electroporation and selected as described (31). Drug resistant cells were flow cytometrically sorted for positive expression of cell surface CD1d, and maintained in culture medium (RPMI 1640 medium; 2mM L-glutamine; 100µg/ml each of Penicillin and Streptomycin from Mediatech, Herndon, VA; 5% Fetal Bovine Serum; 10% Bovine Calf Serum from Hyclone, Logan, UT). For the 3023-d line the culture medium was supplemented with 0.5mg/ml G418 sulfate (Mediatech) and 0.5µg/ml puromycin (Sigma), and for the 2001-d cells 10µg/ml mycophenolic acid and 7.6µg/ml xanthine (both from Sigma) were also included. NKT cell clones were established as previously described (32), and maintained in T cell medium (RPMI 1640 medium; 2mM L-glutamine; 100µg/ml each of Penicillin and Streptomycin; 10% Fetal Bovine Serum; 5% Bovine Calf Serum; 5% human AB serum from Gemini, West Sacramento, CA; and 400 U/ml recombinant human IL-2 from Chiron, Emeryville, CA), with periodic re-stimulation by irradiated allogeneic feeder PBMCs and PHA as described (32).

Flow cytometry. Flow cytometric analysis was performed using Alexa-647 conjugated anti-human CD1d (clone CD1d42.1), Alexa-633 conjugated anti-human MHC class II (clone L243), Alexa-488 conjugated anti-human MHC class I (clone W6/32), or Alexa-647, 633, or 488 conjugated negative control mAbs MOPC21 (IgG1) or UPC10 (IgG2a), prepared according to the manufacturer's protocol (Invitrogen/Molecular Probes, Carlsbad CA). Samples were stained with the indicated specific mAbs or negative controls for 30 minutes on ice, then washed and

resuspended in FACS buffer (1mg/ml PBS/BSA containing 10 μ g/ml DAPI). Live cells were gated by forward and side scatter and exclusion of DAPI.

Preparation of lipid antigens. The glycolipid antigens α -GalCer, and GalGalCer were prepared from D-lyxose as described (33), and dissolved in DMSO at a concentration of 100 μ g/ml, and stored frozen in glass vials at -20°C . Prior to use, the lipids were sonicated in a heated water bath for 15 minutes at 37°C .

Antigen presentation assays. NKT cell clones (5×10^4 /well) were incubated with APCs (5×10^4 /well), or in plates coated with 0.5 μ g/ml human CD1d-Fc fusion protein and pulsed with 10 ng/ml of the indicated antigen as described (32). Assays were performed using 200 μ l/well of T cell medium lacking IL-2, and incubated at 37°C and 5% CO_2 for 16 hours. Where indicated, the APCs were pulsed with the indicated concentration of α -GalCer, or GalGalCer, or an equivalent volume of DMSO alone, for the indicated amount of time. Where noted the APCs were pretreated with 40 μ g/ml chloroquine dissolved in culture medium for two hours, then GalGalCer lipid antigen was added for the indicated pulse period in presence of chloroquine. All assays were performed in triplicate. Supernatants were tested for GM-CSF concentration by a commercial ELISA (Biolegend, San Diego, CA), and quantitated by comparison to recombinant human GM-CSF standards (Peprotech, Rockyhill, NJ).

Immunofluorescence microscopy. Logarithmically growing cells were incubated on lysine-coated slides for 10 minutes at 37°C , then fixed in 2% paraformaldehyde in PBS for 30 minutes at room temperature. The cells were then permeabilized with Cytotfix/Cytoperm Buffer (BD

Biosciences, San Diego, CA) for 30 minutes at room temperature, and stained at room temperature with 10 μ g/ml each of Alexa-647-conjugated anti-CD1d (CD1d42.1) or mouse IgG1 negative control mAb, and FITC-conjugated anti-LAMP-1 (H4A3) or mouse IgG1 negative control mAbs (BD Biosciences, San Diego, CA). The cells were analyzed by confocal microscopy of 0.3 μ m thick sections, using a Carl Zeiss LSM 510 Confocal Fluorescence Microscope. Z-stacks were created from the sections and color images were overlaid using LSM Zeiss software.

Determination of CD1d internalization by cell surface biotinylation. APCs (7 \times 10⁷ each) were labeled for 30 minutes on ice with 0.4 mg/ml Sulfo-NHS-SS-Biotin (Pierce, Rockford, IL), then washed and resuspended in culture medium and incubated for the indicated times at 37°C to allow protein internalization from the cell surface. The cells were then treated with 100 mM DTT for 20 minutes on ice to remove the remaining surface biotin. Residual surface biotin was blocked by addition of 0.5 mg/ml recombinant streptavidin (Pierce), followed by addition of 10 μ g/ml free biotin to block the unbound streptavidin sites. The cells were then lysed in 1% Triton X-100, and lysates were tested by ELISA, using a CD1d-specific capture mAb (CD1d42.1), followed by direct detection with streptavidin-AP (Zymed, S. San Francisco, CA) to detect biotinylated CD1d. Total CD1d was assessed by lysing the APCs in 1% Triton X-100 prior to surface biotinylation, capturing the CD1d by plate-bound CD1d42.1 mAb, and detecting bound CD1d using biotin-labeled rat anti-human β_2 -microglobulin antibody (2 μ g/ml, Dako Scientific, Carpinteria, CA). Specificity of the ELISA was confirmed using a negative control mAb instead of the anti-CD1d capture mAb, and by testing lysates from CD1d untransfected 3023 and 2001 cells. All assays were performed in triplicate.

Determination of CD1d internalization or re-emergence by flow cytometry. APCs were incubated with 10µg/ml unlabeled anti-CD1d mAb (clone CD1d42.1), then washed and resuspended in warm culture medium and incubated at 37°C for the indicated times. The cells were then washed again, fixed in cold 2% paraformaldehyde in PBS, and labeled with 10µg/ml FITC conjugated goat anti-mouse antibody (BD Biosciences) prior to analysis by flow cytometry. For CD1d re-emergence assays, the cells were treated with 50µg/ml cycloheximide for 30 minutes to prevent new protein synthesis, then cell surface CD1d was blocked with unlabeled CD1d42.1 mAb as described above. The cells were incubated for the indicated times at 37°C in culture medium, then washed and fixed with paraformaldehyde, and unblocked CD1d on the cell surface was detected with PE-conjugated CD1d42.1 mAb (BD Biosciences).

Ii chain knock-down by siRNA. Annealed siRNA constructs were obtained from Ambion, Austin, TX. Sense strand sequences used were as follows: GGCUUUCCAUCUGGUGAtt; CCAAGUCGGAACAGCAGAUtt; CCUUAUCUCCAACAAUGAGtt; and non-specific siRNA was used as a negative control. 10-20µg of each siRNA was mixed with 100µl Nucleofector Solution V (Amara, Gaithersburg, MD), and transfected into 5x10⁶ cells using the A24 program of the Nucleofector electroporator device. The cells were then transferred into 5ml culture medium containing 20% FBS, and cultured for three days at 37°C and 5% CO₂ before use.

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References

1. Brigl, M. & Brenner, M. B. (2004) *Annu Rev Immunol* **22**, 817-90.
2. Calabi, F., Jarvis, J. M., Martin, L. & Milstein, C. (1989) *Eur J Immunol* **19**, 285-92.
3. Dascher, C. C. & Brenner, M. B. (2003) *Trends Immunol* **24**, 412-8.
4. Bendelac, A., Lantz, O., Quimby, M. E., Yewdell, J. W., Bennink, J. R. & Brutkiewicz, R. R. (1995) *Science* **268**, 863-5.
5. Kronenberg, M. (2005) *Annu Rev Immunol* **23**, 877-900.
6. Mattner, J., Debord, K. L., Ismail, N., Goff, R. D., Cantu, C., 3rd, Zhou, D., Saint-Mezard, P., Wang, V., Gao, Y., Yin, N., Hoebe, K., Schneewind, O., Walker, D., Beutler, B., Teyton, L., Savage, P. B. & Bendelac, A. (2005) *Nature* **434**, 525-9.
7. Zajonc, D. M., Cantu, C., 3rd, Mattner, J., Zhou, D., Savage, P. B., Bendelac, A., Wilson, I. A. & Teyton, L. (2005) *Nat Immunol* **6**, 810-8.
8. Koch, M., Stronge, V. S., Shepherd, D., Gadola, S. D., Mathew, B., Ritter, G., Fersht, A. R., Besra, G. S., Schmidt, R. R., Jones, E. Y. & Cerundolo, V. (2005) *Nat Immunol* **6**, 819-26.
9. Dougan, S. K., Salas, A., Rava, P., Agyemang, A., Kaser, A., Morrison, J., Khurana, A., Kronenberg, M., Johnson, C., Exley, M., Hussain, M. M. & Blumberg, R. S. (2005) *J Exp Med* **202**, 529-39.
10. Zhou, D., Cantu, C., 3rd, Sagiv, Y., Schrantz, N., Kulkarni, A. B., Qi, X., Mahuran, D. J., Morales, C. R., Grabowski, G. A., Benlagha, K., Savage, P., Bendelac, A. & Teyton, L. (2004) *Science* **303**, 523-527.
11. Kang, S. J. & Cresswell, P. (2004) *Nat Immunol* **5**, 175-81.
12. Prigozy, T. I., Naidenko, O., Qasba, P., Elewaut, D., Brossay, L., Khurana, A., Natori, T., Koezuka, Y., Kulkarni, A. & Kronenberg, M. (2001) *Science* **291**, 664-7.
13. De Silva, A. D., Park, J. J., Matsuki, N., Stanic, A. K., Brutkiewicz, R. R., Medof, M. E. & Joyce, S. (2002) *J Immunol* **168**, 723-33.
14. Bonifacino, J. S. & Traub, L. M. (2003) *Annu Rev Biochem* **72**, 395-447.
15. Sugita, M., Cao, X., Watts, G. F., Rogers, R. A., Bonifacino, J. S. & Brenner, M. B. (2002) *Immunity* **16**, 697-706.
16. Cernadas, M., Sugita, M., van der Wel, N., Cao, X., Gumperz, J. E., Maltsev, S., Besra, G. S., Behar, S. M., Peters, P. J. & Brenner, M. B. (2003) *J Immunol* **171**, 4149-55.
17. Lawton, A. P., Prigozy, T. I., Brossay, L., Pei, B., Khurana, A., Martin, D., Zhu, T., Spate, K., Ozga, M., Honing, S., Bakke, O. & Kronenberg, M. (2005) *J Immunol* **174**, 3179-86.
18. Sugita, M., Cernadas, M. & Brenner, M. B. (2004) *Curr Opin Immunol* **16**, 90-5.
19. Kang, S. J. & Cresswell, P. (2002) *Embo J* **21**, 1650-60.
20. Jayawardena-Wolf, J., Benlagha, K., Chiu, Y. H., Mehr, R. & Bendelac, A. (2001) *Immunity* **15**, 897-908.
21. Cresswell, P. (1994) *Annu Rev Immunol* **12**, 259-93.
22. Chiu, Y. H., Park, S. H., Benlagha, K., Forestier, C., Jayawardena-Wolf, J., Savage, P. B., Teyton, L. & Bendelac, A. (2002) *Nat Immunol* **3**, 55-60.
23. Kawano, T., Cui, J., Koezuka, Y., Toura, I., Kaneko, Y., Motoki, K., Ueno, H., Nakagawa, R., Sato, H., Kondo, E., Koseki, H. & Taniguchi, M. (1997) *Science* **278**, 1626-9.

24. DeMars, R., Rudersdorf, R., Chang, C., Petersen, J., Strandtman, J., Korn, N., Sidwell, B. & Orr, H. (1985) *PNAS* **82**, 8183-8187.
25. Ortiz, L., Demick, K. P., Petersen, J. W., Polka, M., Rudersdorf, R. A., Van der Pol, B., Jones, R., Angevine, M. & DeMars, R. (1996) *J Immunol* **157**, 4554-67.
26. Roberts, T. J., Sriram, V., Spence, P. M., Gui, M., Hayakawa, K., Bacik, I., Bennink, J. R., Yewdell, J. W. & Bratkiewicz, R. R. (2002) *J Immunol* **168**, 5409-14.
27. Zhou, D., Mattner, J., Cantu, C., 3rd, Schrantz, N., Yin, N., Gao, Y., Sagiv, Y., Hudspeth, K., Wu, Y. P., Yamashita, T., Teneberg, S., Wang, D., Proia, R. L., Lavery, S. B., Savage, P. B., Teyton, L. & Bendelac, A. (2004) *Science* **306**, 1786-9.
28. Exley, M., Garcia, J., Balk, S. P. & Porcelli, S. (1997) *J. Exp. Med.* **186**, 109-20.
29. Pierre, P., Turley, S. J., Gatti, E., Hull, M., Meltzer, J., Mirza, A., Inaba, K., Steinman, R. M. & Mellman, I. (1997) *Nature* **388**, 787-92.
30. Greco, O., Marples, B., Dachs, G. U., Williams, K. J., Patterson, A. V. & Scott, S. D. (2002) *Gene Ther* **9**, 1403-11.
31. Gumperz, J. E. (2000) *Methods Mol Biol* **121**, 49-60.
32. Brigl, M., Bry, L., Kent, S. C., Gumperz, J. E. & Brenner, M. B. (2003) *Nat Immunol* **4**, 1230-7.
33. Yu, K. O., Im, J. S., Molano, A., Dutronc, Y., Illarionov, P. A., Forestier, C., Fujiwara, N., Arias, I., Miyake, S., Yamamura, T., Chang, Y. T., Besra, G. S. & Porcelli, S. A. (2005) *Proc Natl Acad Sci U S A* **102**, 3383-8.

Figure Legends

Figure 1. Analysis of antigen presenting molecule expression by the 3023-d and 2001-d cell lines. **A)** Flow cytometric analysis of invariant chain (Ii), MHC class II (DR), and CD1d expression. Filled histograms show specific antibody staining, open histograms show negative controls. Ii staining was performed after permeabilization of the cells to detect intracellular molecules, MHC class II and CD1d staining were performed on unpermeabilized cells to detect cell surface expression. **B)** Semi-quantitative analysis of CD1d mRNA levels. Total RNA was prepared from equal numbers of 3023-d and 2001-d cells, and reverse transcribed to produce total cDNA. PCR was performed on dilutions of the cDNA as shown, using primers specific for human CD1d or GAPDH, and the products analysed by agarose gel electrophoresis. A plasmid containing human CD1d cDNA was used as a positive control (far right lane).

Figure 2. NKT cell responses to auto-antigens and α -GalCer presented by the 3023-d and 2001-d cell lines. **A)** Autoreactive GM-CSF secretion by an NKT cell clone in response to the indicated APCs is plotted on the left y-axis (open bars), and the mean fluorescence intensity (mfi) of CD1d cell surface staining is shown on the right y-axis (filled bars). **B)** GM-CSF secretion by a CD4⁺ and a CD4⁻ NKT cell clone in response to 2001-d (filled squares) and 3023-d cells (open triangles) treated for 16 hours with the indicated concentrations of α -GalCer. The results are shown as the fold increase in GM-CSF production over the autoreactive responses. **C)** Cytokine secretion by NKT cell clones in response to 2001-d and 3023-d cells that were pulsed for the indicated times with 50ng/ml α -GalCer. All assays were performed in triplicate, and the standard deviations of the means are shown as error bars. Similar results were observed in multiple independent experiments using a total of 6 different NKT cell clones.

Figure 3. Access of CD1d molecules to lysosomal antigens in the 3023-d and 2001-d cell lines.

A) Confocal microscopic analysis of CD1d intracellular localization. Permeabilized cells were stained with anti-CD1d (red) and LAMP-1 (green). The images shown are overlays of the CD1d and LAMP-1 staining, and co-localization is indicated by yellow color. **B)** Response of an NKT cell clone to α -GalCer and GalGalCer lipids loaded into recombinant CD1d molecules in vitro. **C)** GM-CSF production by an NKT cell clone in response to 2001-d (filled squares) and 3023-d (open triangles) cells that were pulsed with GalGalCer for the indicated times. Solid lines show GalGalCer presentation by mock-treated, and dashed lines show chloroquine treated cells. Results are given as the fold increase in GM-CSF production over the autoreactive responses. **D)** Autoreactive responses of an NKT cell clone to chloroquine and mock treated 3023-d and 2001-d cells. Assays were performed in triplicate and error bars represent the standard deviations of the mean. The results shown are representative of 3 independent experiments.

Figure 4. Rate of CD1d cell surface trafficking in the 3023-d and 2001-d cells. **A)** Cell surface proteins were labeled with biotin, then allowed to internalize for the indicated times before the surface biotin was removed. Biotinylated and total CD1d were assayed by ELISA. Results are shown as the biotinylated CD1d normalized by the total CD1d from lysates of the 2001-d (filled squares) or 3023-d (open triangles) cells. **B)** Internalization of CD1d from the cell surface was assessed by flow cytometry by detecting anti-CD1d antibody remaining at the cell surface after the indicated incubation times. The results are shown as the CD1d signal detected at each time point, normalized by the starting signal. **C)** Emergence of CD1d molecules from the endosomal system was analyzed in 3023-d and 2001-d cells by blocking existing cell surface CD1d molecules and staining for unblocked CD1d after the indicated incubation times. The results are

shown as the CD1d signal detected at each timepoint, normalized by the CD1d staining of the unblocked cells.

Figure 5. Effect of siRNA Ii chain knock-down on CD1d expression. **A)** CD1d expression was analyzed by flow cytometry in unpermeabilized (black bars) and permeabilized (grey bars) 3023-d and 2001-d cells. The results are shown as the CD1d signal of negative control or Ii siRNA treated cells, normalized by the CD1d staining in mock-treated cells. The 100% mark is shown as a dashed line. **B)** and **C)** CD1d and MHC class I internalization from the cell surface in negative control and Ii siRNA treated 3023-d and 2001-d cells. Internalization was assessed as described for Figure 4B. The 2001-d cells are represented by squares and the 3023-d cells by triangles; filled symbols show cells that were transfected with negative control siRNA, and open symbols show those that were transfected with siRNA specific for the Ii chain. The results shown are representative of at least 3 independent experiments.

Figure 1

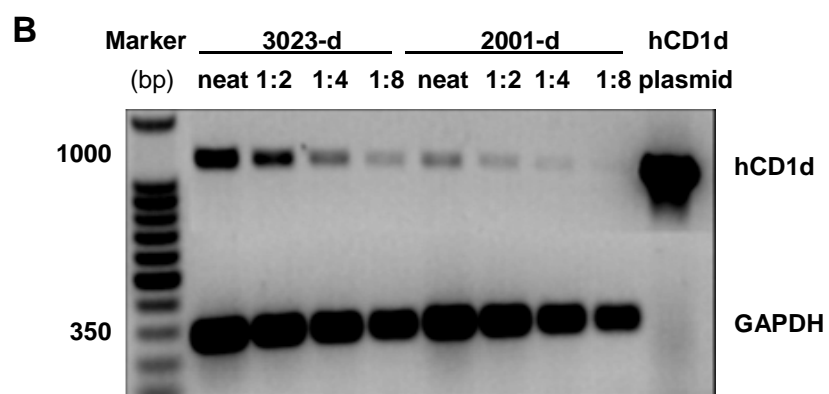
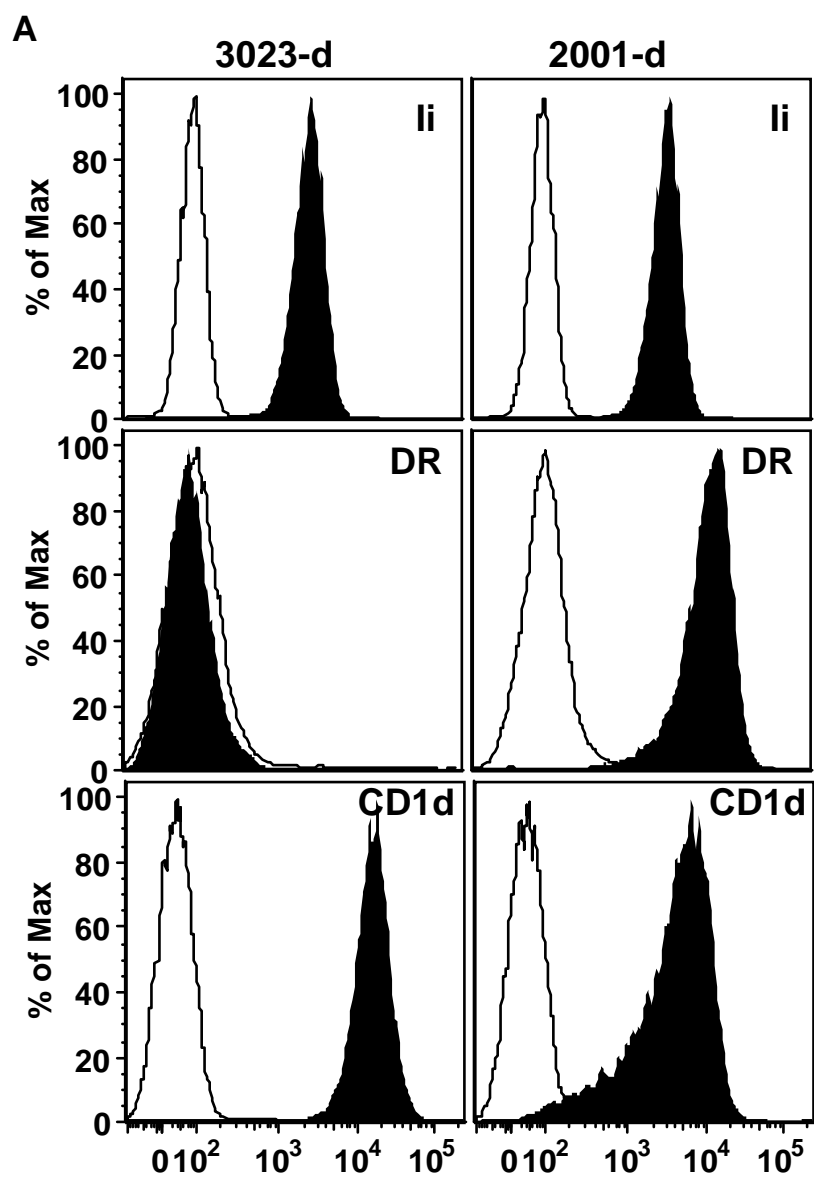
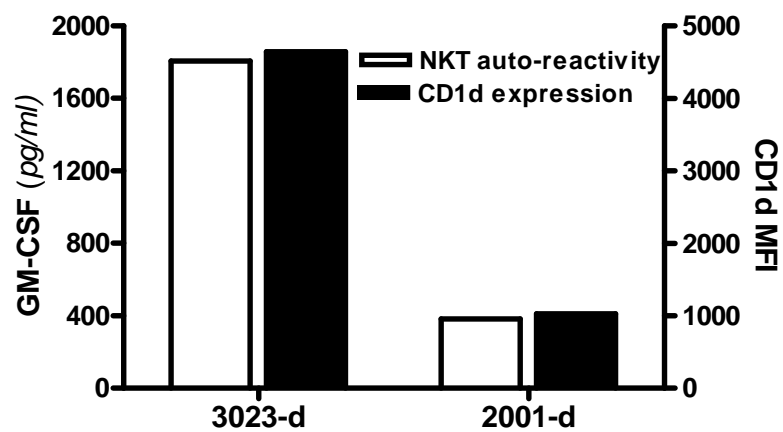
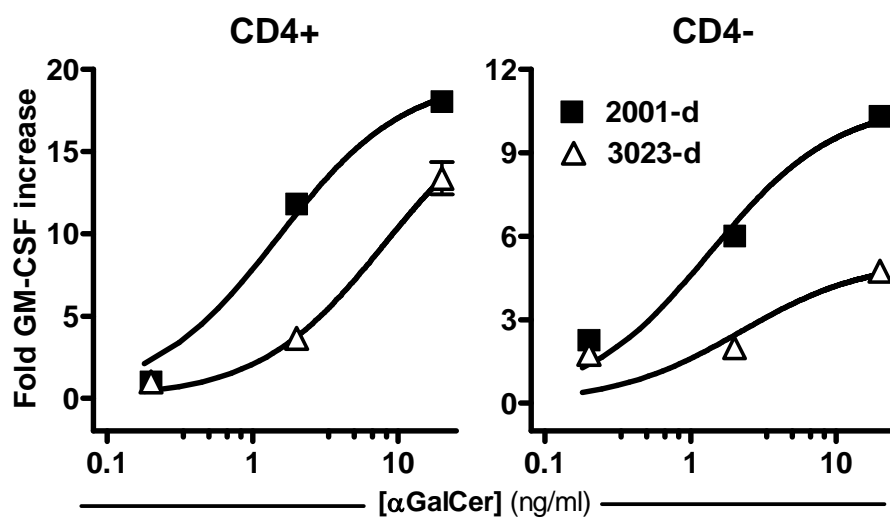


Figure 2

A



B



C

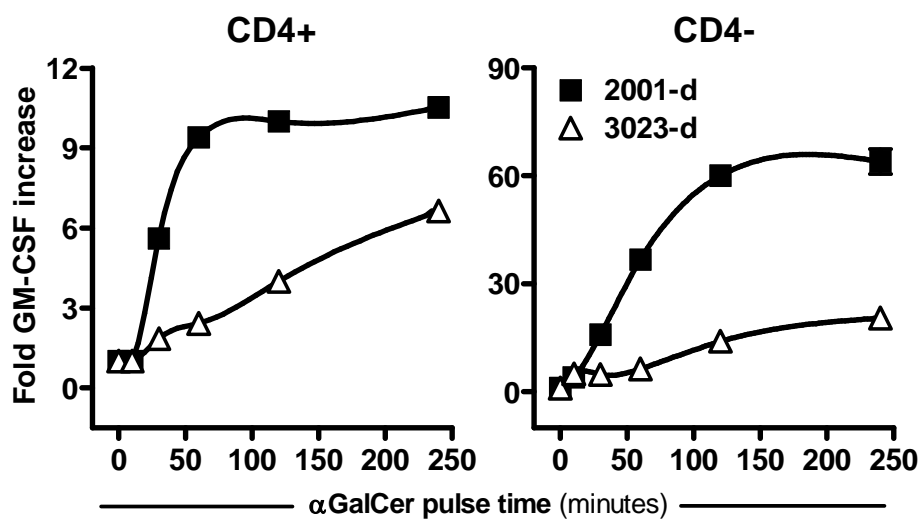


Figure 3A

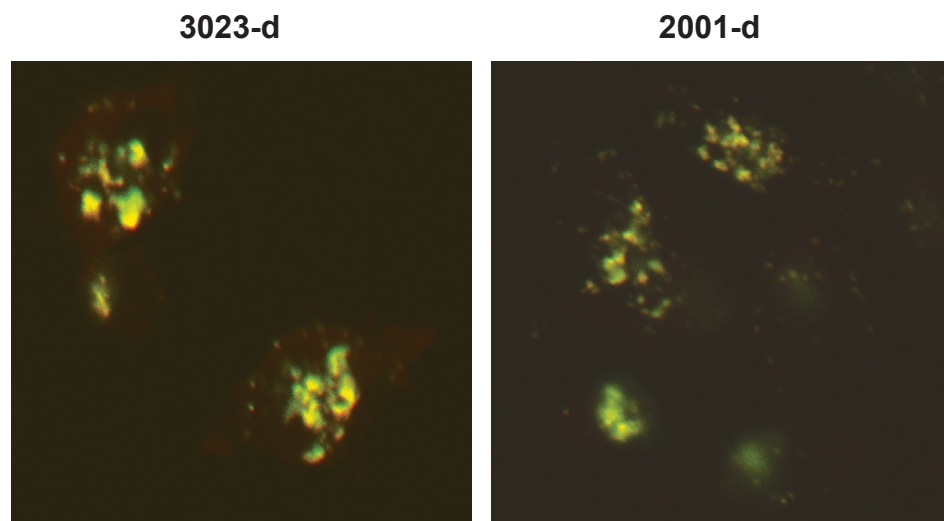
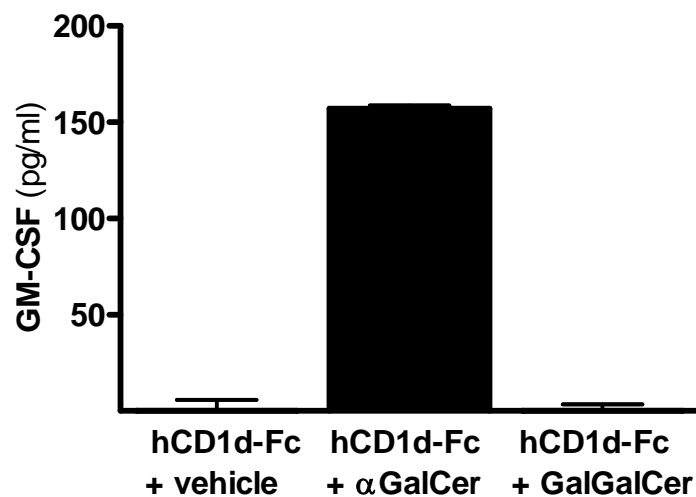
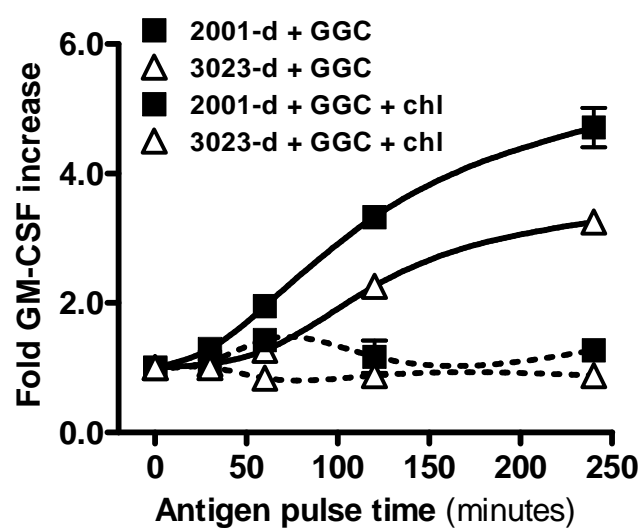


Figure 3

B



C



D

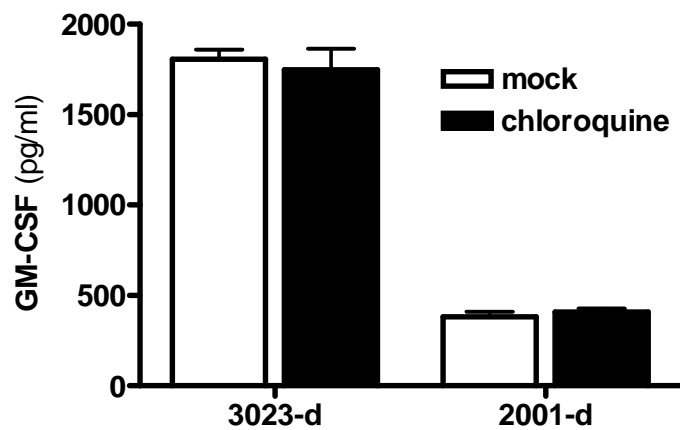


Figure 4

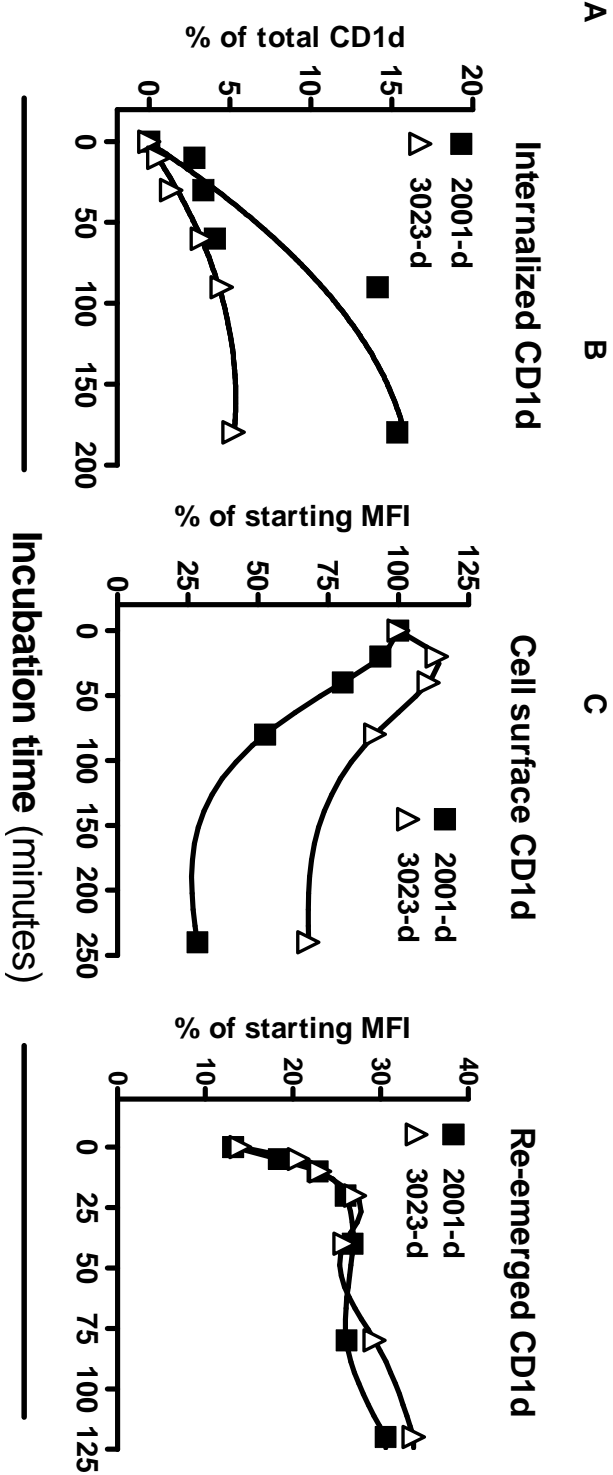
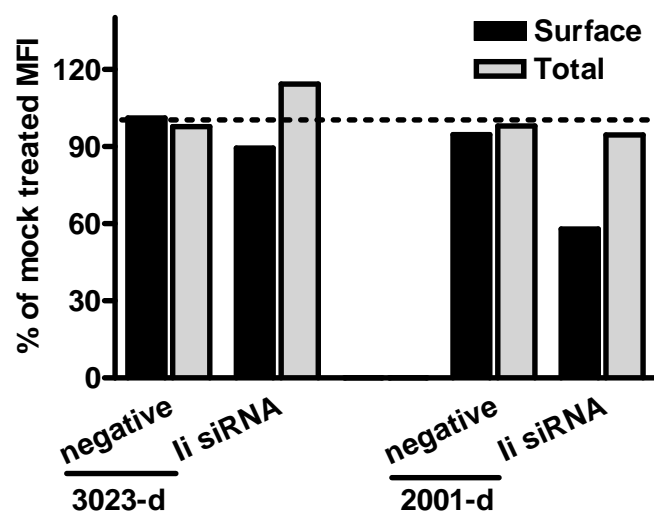
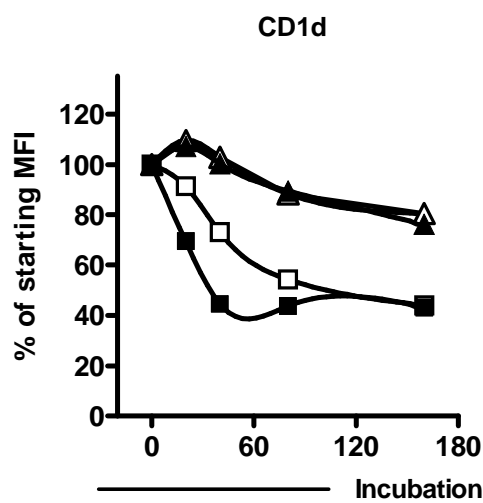


Figure 5

A



B



C

